HEAVY METAL PROSTHETIC GROUPS AND ENZYME ACTION

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TRANSLATED BY
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OXFORD
AT THE CLARENDON PRESS
1949

Oxford University Press, Amen House, London E.C. 4
GLASGOW NEW YORK TORONTO MELBOURNE WELLINGTON
BOMBAY CALCUTTA MADRAS CAPE TOWN
Geoffrey Cumberlege, Publisher to the University

PRINTED IN GREAT BRITAIN

PREFACE

EVER since it has been known that cells respire the chief problem connected with respiration has been to determine which part of the living matter is autoxidizable. If the combustible substances in the cell are not autoxidizable, and if the cell material itself is not, with what then does the molecular oxygen, which is absorbed by the respiring cell, react?

The answer to the problem lies in the autoxidizable ferrous iron complex which is oxidized to ferric iron by molecular oxygen and transformed again to ferrous iron by the reducing action of the cell constituents.

The long road at the end of which this explanation lay started with the experiments of Edmund Davy, who in 1820 discovered the oxidizing action of finely divided platinum—the first model for cell respiration. In 1857 Claude Bernard observed that after cyanide poisoning, venous blood was bright red, and he thus discovered the specific inhibition of cell respiration by cyanide. In 1885 MacMunn using the spectroscope reported in all cells 'from Echinoderms to man throughout the animal kingdom' the presence of histohaematins and he noted their oxidizing actions. In 1896 John Haldane observed by colorimetric analysis of carbonylhaemoglobin that the carbon monoxide appeared to be more firmly bound in winter than in summer and so discovered the photochemical dissociation of carbon monoxide-iron compounds. In 1923 the oxidation of amino-acids adsorbed on blood charcoal was discovered. This was 'iron catalysis on surfaces', catalysis, which like cell respiration, could be non-specifically inhibited by narcotics and specifically by cyanide. In 1926 the discovery was made that carbon monoxide inhibits cell respiration and that light diminishes this action. In 1928 the absorption spectrum of the oxygen transporting enzyme was determined using light of different wavelengths on the carbon monoxide inhibited reaction.

These are the most important stages, the seven pillars on which our knowledge is based. No one who has seen these

experiments will question the existence of oxygen transporting iron—the enzyme which has contributed more than any other to the explanation of life, but which has as yet not been isolated.

In addition to oxygen transporting iron I have also dealt with the oxygen transporting copper complex of the phenol oxidases, the hydrogen producing iron catalyst of the butyric acid bacteria, the heavy metal of yeast zymohexase discovered in 1942, and finally, the heavy metal of the chloroplasts and its function in the photo-reactions discovered in 1944.

Thus it can be recognized that oxygen transporting iron has not only provided the solution to a great biological problem, but is also the key to the understanding of further and no less important aspects of life.

The last chapter does not deal with heavy metals. It is a report on experiments which I finished in the Spring of 1945—the swan song of the Kaiser-Wilhelm-Institut für Zellphysiologie.

O. W.

Berlin-Dahlem, December 1946

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CHAPTER I

DAVY'S MODEL

1. Edmund Davy's experiments

In 1820 Edmund Davy† found that alcohol in aqueous solution is oxidized at room temperature to acetic acid by atmospheric oxygen when a small amount of finely divided platinum is added to the solution. This discovery aroused much interest, as the platinum was reacting under conditions corresponding to those of acetic acid fermentation. Since then, finely divided noble metals have been used as models for oxidizing enzymes and much work has been done in investigating the analogy with biological oxidations. The scientific instinct of such investigators as Berzelius,‡ Schönbein,§ and Bredig‖ persuaded them to regard such an analogy as being more than coincidental. Heavy metal catalysis at surfaces, brought about in model experiments by platinum and in biological processes by iron, has finally been the answer to their questions.

2. Wieland's experiments on Davy's model

In 1912 Heinrich Wieland†† began an investigation of Davy's model with a view to explaining the chemical mechanism of the oxidizing action of platinum. He found that the reaction took place in two stages. In the first stage the metal combined with the hydrogen of the substrate. In the second, molecular oxygen oxidized the hydrogen combined with the metal. If we consider, for example, the oxidation of hydroquinone by molecular oxygen and palladium, then according to Wieland the mechanism would be

$$\text{hydroquinone} + \text{Pd} = \text{quinone} + \text{PdH}_2$$
$$\text{PdH}_2 + \text{O}_2 = \text{Pd} + \text{H}_2\text{O}_2.$$

† Edmund Davy, *Phil. Transact. Royal Soc.* London, 1820, Part I, p. 108.

‡ Jakob Berzelius, *Jahresber. über d. Fortschr. d. phys. Wiss.* **15,** 237 (1836).

§ Christian Friedrich Schönbein, *Journ. prakt. Chem.* **89,** 22, 323 (1863); **105,** 198 (1868).

‖ Bredig *et al.*, *Z. f. physikalische Chem.* **31,** 258 (1899); **37,** 1, 448 (1901).

†† Heinrich Wieland, *Chem. Berichte,* **45,** 484 (1912); **46,** 3327 (1913).

The second of these reactions, the union of hydrogen and oxygen, was known to be catalysed by platinum metals.† Wieland believed that the proof of the first reaction taking place lay in the fact that when he shook hydroquinone with palladium in the absence of oxygen he found that quinone was formed, and that the palladium had combined with an equivalent amount of hydrogen.

In the same way, Wieland showed that many other substances such as glucose could be oxidized by palladium in the absence of oxygen. In this particular case, carbon dioxide as well as hydrogen resulted.

If, however, one examines Wieland's results more closely it is apparent that the oxidations in the absence of oxygen do not proceed beyond the decomposition of a small amount of substrate even when a large excess of palladium is present. Thus 1·8 g. of glucose, after being shaken for 10 hours with 16 g. of palladium, gave only 0·058 g. of carbon dioxide.

Wieland overcame this difficulty with the suggestion that the dehydrogenation was reversible, but it is obvious that this explanation cannot be right in the case of glucose, since the formation of carbon dioxide from glucose is an irreversible process.

3. Personal investigations

In 1924 we applied the manometric technique to the Davy model and were able to study quantitatively‡ the questions which, up till then, had only been dealt with qualitatively. First we investigated the hydrogen–oxygen reaction with palladium. We found that the addition of hydrogen cyanide raised the yield of hydrogen peroxide [found to be only a few per cent. by Traube§] to 60 per cent., and we showed that the equation

$$PdH_2 + O_2 = Pd + H_2O_2$$

expressed the true mechanism of the reaction.

On this basis one should also find hydrogen peroxide in the

† Moritz Traube, *Chem. Berichte*, **22**, 1496 (1889) [p. 1509].
‡ Kanicki Tanaka, *Bioch. Z.* **157**, 425 (1925).
§ Moritz Traube, *Chem. Berichte*, **22**, 1496 (1889).

oxidation of alcohol with palladium, and certainly in the presence of hydrogen cyanide. In no case, however, did we find even a trace of hydrogen peroxide when we shook aqueous solutions of alcohol with palladium.

We concluded, therefore, that the Wieland theory of the Davy model could not be right, and that in the Davy experiment the oxygen had reacted with the metal, the oxidation of alcohol being brought about by palladium oxide. It appears to us that this view is not incompatible with the assumption that it is molecular hydrogen on the palladium which reacts with molecular oxygen since the oxidation of molecular hydrogen and of hydrogen bound chemically to carbon cannot be comparable reactions.

In replying, Wieland† did not accept this point of view. He left unanswered the question as to why hydrogen peroxide is found in the oxidation of molecular hydrogen on palladium and not in the oxidation of alcohol under similar conditions.

4. Experiments of Gillespie and Liu

In 1931, nineteen years after their publication, the Wieland results were re-examined.

Gillespie and Liu‡ reasoned thus. In a saturated solution of hydroquinone, the hydrogen pressure amounts to 10^{-24} atmospheres. On the other hand, palladium forms only a loose combination with hydrogen. Therefore, on thermodynamical grounds metallic palladium cannot dehydrogenate hydroquinone. The quinone formation observed by Wieland must, therefore, have a different explanation.

Wieland prepared his palladium by reduction of the chloride in alkaline solution with formic acid, so that there was a danger of the precipitated palladium containing palladium hydroxide as impurity. In order to avoid this impurity Gillespie and Liu prepared their palladium by another method, namely, by heating palladousammine in a current of hydrogen. Whilst Wieland's palladium immediately formed quinone from hydroquinone in

† Heinrich Wieland and F. G. Fischer, *Chem. Berichte*, **59**, 1180 (1926).
‡ L. J. Gillespie and T. H. Liu, *J. Amer. Chem. Soc.* **53**, 3969 (1931).

the absence of oxygen, no quinone was observed with Gillespie's palladium until oxygen was available. Thus it was shown that Wieland's palladium was not free from oxygen, and that his so-called oxygen-free oxidation was brought about by palladium oxide. This naturally explained the low yields in Wieland's experiments described above.

But there remained unexplained why Wieland found in his oxygen-free oxidations both products of the dehydrogenation, not only the dehydrogenated substrate, but also the hydrogen which had been removed. This question, which was not considered by Gillespie and Liu, is apparently to be answered as follows:

Wieland previously treated with hydrogen the palladium which he used for his dehydrogenation experiments in order to be sure that it contained no oxygen. The hydrogen content of aliquots of the palladium preparation was then determined by heating in a stream of carbon dioxide both before and after the dehydrogenation. The difference gave the hydrogen which had been removed by the dehydrogenation of the substrate.

Let us see how an impurity of palladium oxide would affect the reaction under these conditions. By heating in the carbon dioxide the palladium oxide and the hydrogen would react with one another, and less hydrogen would be found than the preparation originally contained, but after the palladium oxide had been used for a dehydrogenation—for example, the oxidation of hydroquinone to quinone—then on heating in the carbon dioxide less palladium oxide would be available to react with the hydrogen, so that the hydrogen content of the palladium would have apparently increased in the course of the dehydrogenation, and moreover by an amount equivalent to the quantity of substrate which had been dehydrogenated. This follows because the reacting substance was palladium oxide in both the oxidation of the substrate and in the oxidation of the hydrogen.

$$PdO + \text{hydroquinone} = Pd + \text{quinone} + H_2O$$

$$PdO + H_2 = Pd + H_2O.$$

5. Application to biological oxidation processes

The oxygen-free palladium and its oxidizing action were the basis of the well-known 'Theory of Biological Oxidation' which Wieland† advanced in 1912, and which was considered for a decade as offering the solution to the problem of biological oxidation. The oxidation enzymes according to Wieland are not like palladium in being capable of removing hydrogen from the organic substrate, but they activate the hydrogen so that it will react with molecular oxygen. For example, if we let H* represent the activated hydrogen of the substrate, e.g. sugar, amino-acid, or fat, then the general equation for a biological oxidation would be:

$$R\begin{matrix}H^* \\ H^*\end{matrix} + O_2 = R + H_2O_2.$$

In 1923 I challenged the theory because in so far as it concerned the reaction mechanism of molecular oxygen it was incompatible with the oxygen transfer brought about by iron.‡ The theory was also incompatible with the oxygen-transporting mechanism associated at a later date with copper and with the yellow enzymes, in fact, with all oxygen-transporting enzymes. When an enzyme transports oxygen the molecular oxygen reacts with the enzyme and not with the substrate. The theory of biological oxidation has proved to be just as erroneous as the conclusions drawn from the palladium experiments which gave rise to it.

Wieland§ has not recanted either in respect of the palladium experiments or of his theory of biological oxidation. He has, however, more and more emphasized that it was immaterial what the oxygen and what the substrate reacted with. All that mattered was that the oxidation of the substrate took place, not by the uptake of oxygen, but by the loss of hydrogen. And so the theory, the object of which had been to define the reaction mechanism of the molecular oxygen, became pointless.

† H. Wieland, *Chem. Berichte*, **45**, 484 (1912); **46**, 3327 (1913); *Ergebnisse der Physiologie*, **20**, 477 (1922); *Liebigs Annalen*, **445**, 181 (1925).

‡ O. Warburg, *Bioch. Z.* **142**, 518 (1923).

§ A. Bertho, *Ergebnisse der Enzymforschung*, **2**, 204 (1933).

Oxidation by dehydrogenation was no theory, but a generally recognized fact which no one would dispute. As far back as 1862 Adolf Strecker† had observed that alloxan and amino-acids react together when in aqueous solution at room temperature. 'Indem das Alloxan durch Aufnahme von Wasserstoff in Alloxanthin übergeht, erfolgt die Oxydation des Alanins und des Leucins zu Aldehyde, Kohlensäure und Ammoniak.' In 1911 Wilhelm Traube‡ had shown that quinone can be used as the dehydrogenator instead of alloxan. And from a biological point of view even more important was the discovery in 1885 by Paul Ehrlich§ that methylene blue in the living cell is converted by the uptake of hydrogen into leuco-methylene blue, acting therefore as an oxidizing agent by removing hydrogen.

If the Wieland theory had been based on nothing more than these results, science would have escaped the ten-year-long polemic over oxygen transporting iron. Oxygen transporting iron and oxidation by dehydrogenation are not incompatible. On the contrary, 'oxidation by dehydrogenation' can almost be regarded as a consequence of oxygen transporting iron, for when molecular oxygen has oxidized the ferro iron to the ferri state it has played its part in respiration. All further oxidations must proceed anaerobically and these oxidations can only be brought about through loss of electrons or loss of hydrogen.

† Adolph Strecker, *Liebigs Annalen*, **123**, 363 (1862).
‡ Wilhelm Traube, *Chemische Berichte*, **44**, 3145 (1911).
§ Paul Ehrlich, *Das Sauerstoffbedürfnis des Organismus*. Berlin, 1885.

CHAPTER II

ACTION OF NARCOTICS ON CHEMICAL PROCESSES IN THE CELL

1. Researches of Claude Bernard

In his lectures on biological processes Claude Bernard† described how fermentation by living yeast cells is inhibited by chloroform and restored on washing out the chloroform. He also observed the same phenomena with oxygen evolution in irradiated living plants. Claude Bernard thereby discovered that narcotics reversibly inhibit chemical processes in the cell.

Claude Bernard also believed that, in contrast to fermentation and carbon dioxide assimilation, oxygen respiration was not inhibited by narcotics. This was an error caused by faulty technique.

2. Limiting concentrations of narcotics

E. Overton‡ determined for a large number of narcotics the concentrations which were just enough to inhibit the movement of tadpoles swimming in water. Overton called these concentrations, which were present in partition equilibrium with the cells, the limiting narcotic concentrations and found, for example, the following values:

	Limiting concentration of narcotic mole/litre
Methyl alcohol	0·57
Ethyl alcohol	0·29
Propyl alcohol	0·11
Butyl alcohol	0·038
Amyl alcohol	0·023
Methylurethane	0·27
Ethylurethane	0·041
Phenylurethane	0·0006
Acetone	0·26
Methylpropylketone	0·019
Methylphenylketone	0·0009

† Claude Bernard, *Leçons sur les phénomènes de la vie, communs aux animaux et aux végétaux*, Paris, 1885 (pp. 276–9).

‡ E. Overton, *Studien über die Narkose*, Jena, 1901.

The movement of protozoa and the streaming of plasma in plant cells are also reversibly inhibited by narcotics. The limiting narcotic concentrations for such inhibitions are, however, according to Overton, six to ten times as great as those for the paralysis of tadpoles. A narcotic which paralyses tadpoles only in saturated solution will therefore not narcotize protozoa or plant cells. At the conclusion of his work Overton asks the question whether narcotics inhibit chemical processes in the cells. Overton confirmed the observation of Claude Bernard that the oxygen evolution on exposing green plant cells to light is inhibited by narcotics. Whether also the aerobic respiration is also inhibited was, according to Overton, uncertain.

3. The half-value narcotic concentration

We have quantitatively investigated the action of narcotics on metabolic processes in cells, using manometric methods. We have confirmed thereby Claude Bernard's observation relating to fermentation and carbon dioxide assimilation. We also found that the aerobic respiration is inhibited by narcotics. If the half-value concentration is defined as that concentration of a narcotic which reduces the rate of a chemical process to half its value, then the respiration—and fermentation—inhibiting half-value concentrations are generally the same. The assimilation inhibiting half-value concentration is, however, considerably smaller than that for the respiration or fermentation. It is worthy of note that this is independent of the nature of the cell. If the same physical explanation—a change in the nature of the surfaces—is valid for all narcotic phenomena, then the variability of the half-value concentration figures would show that the inhibition of each biological process is a problem in itself. In no case, however, is the inhibition of the oxygen respiration the primary cause of any narcotic phenomena.

For the respiration of avian red blood cells the following half-value concentrations were found:

	Half-value† concentration • mole/litre		*Half-value† concentration mole/litre*
Methyl alcohol	5·0	Acetal	0·14
Ethyl alcohol	1·6		
Propyl alcohol	0·8	Acetone	0·90
Butyl alcohol	0·15	Methylpropylketone	0·17
Amyl alcohol	0·045	Methylphenylketone	0·014
Methylurethane	1·3		
Ethylurethane	0·33	Acetonitrile	0·85
Propylurethane	0·13	Propionitrile	0·36
Butylurethane	0·043	Valeronitrile	0·06
Phenylurethane	0·0043		
Methylal	0·60	Dimethylurea	1·4
		Diethylurea	0·52
		Phenylurea	0·018

For the fermentation of yeast cells‡ the following values were obtained:

	Half-value concentration mole/litre
Ethylurethane	0·41
Propylurethane	0·19
Butylurethane	0·06
Phenylurethane	0·006
Propionitrile	0·73
Valeronitrile	0·084
Methylpropylketone	0·23
Methylphenylketone	0·014
Dimethylurea	1·0
Diethylurea	0·43
Phenylurea	0·037

For the respiration and assimilation of the alga, Chlorella,§ we found the following half-value concentrations (mole per litre).

	Respiration	*Assimilation*
Methylurethane	1·2	0·40
Ethylurethane	0·78	0·22
Propylurethane	0·10	0·05
Butylurethane	0·043	0·017
Amylurethane	0·032	0·012
Phenylurethane	0·006	0·0005

† O. Warburg, *Z. f. physiolog. Chem.* **69**, 452 (1910); **70**, 413 (1911); **71**, 479 (1911).

‡ A. Dorner, *Z. f. physiolog. Chemie*, **81**, 99 (1912).

§ O. Warburg, *Bioch. Z.* **100**, 230 (1919); **103**, 188 (1920).

4. Homologous series

The following amines are examples of substances whose inhibiting action is not a narcotic inhibition.† We found the following values for the respiration of avian red blood cells.

	Half-value concentration mole/litre
Ammonia	0·02
Monomethylamine	0·01
Dimethylamine	0·01
Trimethylamine	0·02
Triethylamine	0·01
Propylamine	0·01
Allylamine	0·01
Amylamine	0·01
Benzylamine	0·02
Piperidine	0·01

From the results for such a homologous series it can be concluded that the amino group determines the inhibitory action. An increase in potency as a homologous series is ascended is characteristic of narcotic action, and serves primarily to define such an action.

5. Choice of narcotic

Since for the inhibition of a chemical action in the cell a higher narcotic concentration than is necessary for producing nerve paralysis is required, we find that narcotics exist, the solubility of which is sufficient to cause cerebral narcosis, but which are not capable of inhibiting respiration and fermentation. For the inhibition of chemical reactions in the cell, therefore, the last-mentioned compounds of the Overton series are unsuitable. Also such substances as chloroform, ether, and toluene are unsuitable for reversible inhibition of chemical reactions in the cell because they plasmolize the cells at concentrations which lie near the respiration-inhibiting or fermentation-inhibiting values. They cause, therefore, irreversible inhibition. Chloroform and toluene in saturated solution are not narcotics, but antiseptics.

† Eduard Grafe, *Z. f. physiolog. Chem.* **79**, 421 (1912).

I have dealt first with the narcotic inhibition of chemical reactions in the cell because in what follows the specific inhibitions play a large part. Specific inhibitions cannot be recognized if one is unable to differentiate them from narcotic inhibitions.

CHAPTER III

ACTION OF HYDROGEN CYANIDE ON CHEMICAL REACTION IN THE CELL

1. Researches of Claude Bernard

CLAUDE BERNARD† discovered that cyanide inhibits cell respiration and that the inhibition is reversible.

En faisant respirer de l'acide prussique à un animal, on voit que son sang veineux devient rutilant. Quelquefois, le lendemain de la mort on retrouve encore la même coloration dans le sang du cœur.

Bien que la quantité de l'acide respirée soit extrêmement minime, le chien est très malade. Si d'ici quelques instants il ne succombe pas rapidement, il reviendra à une santé parfaite.

Mitscherlich, devant qui l'on parlait un jour des effets de l'acide prussique, regardait son action comme très-mystérieuse et attachait à son explication une grande importance.

Die hellrote Farbe des Venenblutes [according to Hoppe-Seyler's‡ comment on the work of Claude Bernard] kann nur darin ihre Ursache haben, dass das Blut auf seinem Weg durch die Gefässe keinen Sauerstoff an die Gefässwände abgibt, und hierfür ist keine andere Ursache denkbar, als dass die Oxydation suspendiert ist.

2. A simple experiment to demonstrate the inhibition of respiration by cyanide§

Three little tubes of the type shown in Fig. 1 are filled with defibrinated chicken blood saturated with air. Tubes 1 and 2 serve as controls. Hydrogen cyanide is added to tube 3 so that the concentration is 1/1000 N.

Tube 1 is placed in ice-water, 2 and 3 in water at 38°. After an hour tubes 2 and 3 are also cooled in ice-water, and the contents of all three tubes well mixed with the help of a glass bead.

On comparing the tubes the contents of 1 are seen to be bright red since the chicken blood cells do not respire at 0°. Tube 2 is dark red because the chicken blood cells respire at 38°.

† Claude Bernard, *Leçons sur les effets des substances toxiques*, Paris, 1857.
‡ Hoppe-Seyler, *Mediz.-Chem. Untersuchungen*, Berlin, 1866, Part I, p. 10.
§ O. Warburg, *Z. f. physiolog. Chemie*, **70**, 413 (1911).

Tube 3 is bright red like 1, since the N/1000 hydrogen cyanide inhibits the respiration. The criticism that the hydrogen cyanide might possibly react with oxyhaemoglobin and make it incapable of liberating its oxygen is not tenable since the oxyhaemoglobin was present in about ten times excess over the cyanide. Actually, hydrogen cyanide does react with methaemoglobin, but under the conditions of this experiment not with oxyhaemoglobin or haemoglobin.

FIG. 1

If, after the comparison of the colours of the tubes is made,† the oxygen of the oxyhaemoglobin is determined by setting it free with ferricyanide according to the Haldane method, it is found that in tube 2 about one-third of the oxygen, and in tubes 1 and 3 no oxygen, has been respired. N/1000 hydrogen cyanide therefore completely inhibits the respiration of chicken red blood cells.

3. Ionization of hydrogen cyanide

According to the tables of Landolt–Börnstein the dissociation constants of hydrogen cyanide are:

$$K = \frac{[H^+][CN^-]}{[HCN]}$$

At 18° . .	$4{\cdot}7 \times 10^{-10}$ mole/litre
„ 25° . .	$7{\cdot}2 \times 10^{-10}$ „
„ 40° . .	$15{\cdot}7 \times 10^{-10}$ „

The degree of dissociation at pH 7·5 is therefore:

$$\frac{K}{[H^+]+K}$$

At 18° . .	0·015
„ 25° . .	0·022
„ 40° . .	0·05

It is apparent from this that at physiological pH and temperature any cyanide added to a solution is really present as undissociated hydrogen cyanide.

4. The Bunsen absorption coefficient of hydrogen cyanide

If x c.mm. cyanide are added from the side tube of a manometric measuring vessel to excess acid contained in the vessel

† Morizo Onaka, *Z. f. physiolog. Chemie*, **70**, 433 (1911); **71**, 193 (1911).

proper, and a pressure h is developed, then the Bunsen absorption coefficient α of the hydrogen cyanide can be calculated from the equation

$$h = P_0 \frac{x}{V_G \frac{273}{T} + V_F \alpha}.\dagger$$

We found $\alpha = 240$ at 20° and in M/2 KH_2PO_4 solution. Knowing α, the partial pressure of the hydrogen cyanide in contact with the aqueous solution can be calculated. For example, taking $\alpha = 240$ (at 20° in M/2 phosphate solution)

	Partial pressure of HCN
10^{-4} N HCN in solution . .	$h = 0{\cdot}9 \times 10^{-5}$ atmos.
10^{-3} N ,, ,, . .	$h = 0{\cdot}9 \times 10^{-4}$,,
10^{-2} N ,, ,, . .	$h = 0{\cdot}9 \times 10^{-3}$,,

It is obvious that the partial pressure of hydrogen cyanide measured manometrically is negligible when the concentration of the free hydrogen cyanide in the aqueous solution is not greater than 10^{-3} N. In spite of this, however, the pressure of hydrogen cyanide with very dilute solutions is great enough to permit of the complete removal of hydrogen cyanide from neutral aqueous solutions by aeration. For the same reason hydrogen cyanide will distil from the main vessel into the inner tube of a manometric apparatus, if the inner tube contains potassium hydroxide. H. A. Krebs‡ has therefore advised the addition of enough potassium cyanide to the potassium hydroxide in the inner tube to equilibrate the vapour pressure in the two compartments.

5. Methods of determination

The best method of determining the hydrogen cyanide action is the manometric estimation with paired vessels. The method was described in 1924,§ and was so improved in 1931‖ that it is suitable for the determination in all cases. The assumption is made that the cells introduced into both vessels are of the same

† O. Warburg, *Bioch. Zeitschr.* **189**, 354 (1927), p. 373.
‡ H. A. Krebs, *Bioch. Journ.* **29**, 1620 (1935).
§ O. Warburg, *Bioch. Zeitschr.* **152**, 51 (1924).
‖ O. Warburg *et al.*, *Bioch. Zeitschr.* **242**, 170 (1931).

quantity, a condition which is easily arranged when isolated cells such as yeast, red blood cells, bacteria, or single-cell green algae are being used. The cells are brought to a homogeneous suspension which is added from a pipette to both vessels. When tissue is being investigated, very thin slices are necessary. If the tissue is too tough to be cut, the manometric method is not suitable, and the older and more troublesome methods must be used. This involves, for example, the perfusion of organs together with the determination of the oxygen and carbon dioxide or lactic acid in the inflowing and outflowing blood.

6. Sources of error in experiments with hydrogen cyanide

Hydrogen cyanide can disappear from solutions not only by distillation but also by chemical reaction, for example, by oxidation to cyanic acid, by formation of cyanhydrins, or by combination with methaemoglobin. According as these reactions are fast or slow the effect of the hydrogen cyanide on the cells can be completely masked, or an inhibition followed by a recovery may be observed.

Hydrogen cyanide reacts very quickly with methaemoglobin, a fact to be remembered in all experiments with blood and organs containing blood, particularly the spleen.

Hydrogen cyanide is oxidized in the presence of carbon,† and also, according to Ellinger,‡ by muscle, to give cyanic acid, which is further hydrolysed in acid solution to carbon dioxide and ammonia.

Moreover, cyanhydrin formation, even if the hydrogen cyanide is very dilute, takes place more quickly than one would expect.

In experiments on the oxidation of fructose in salt solution at 38°, H. A. Krebs§ observed that 10^{-3} N hydrogen cyanide at first completely inhibited the oxidation, but that after a few hours the effect disappeared. Cyanide determinations showed that the prussic acid had been removed from the solutions by the formation of fructose cyanhydrin.

† O. Warburg, *Bioch. Zeitschr.* **119**, 134 (1921), p. 161.
‡ Ph. Ellinger, *Z. f. physiolog. Chem.* **154**, 85 (1926).
§ H. A. Krebs, *Bioch. Zeitschr.* **180**, 377 (1926).

An initial inhibition by hydrogen cyanide followed by recovery was also observed by Wieland and Bertho† in the oxidation of alcohol by acetic acid bacteria. The acetaldehyde which is formed reacts with the hydrogen cyanide. When acetaldehyde and N/100 HCN were allowed to react (in the absence of the acetic acid bacteria), after 30 minutes, 65 per cent. of the cyanide had disappeared owing to cyanhydrin formation.

Among other substances which react rapidly with prussic acid at physiological pH and temperature are the following: methylglyoxal, pyruvic acid, glyceraldehyde, glyceraldehyde-phosphate.

7. Relationship between the effect and the concentration of hydrogen cyanide

If hydrogen cyanide reversibly inhibits a biochemical process, we can assume that the hydrogen cyanide forms a dissociating complex with the particular enzyme which brings about the reaction. The cyanide compound of the enzyme is therefore catalytically inactive.

In the simplest case, if Fe denotes the enzyme, then

$$\frac{[\mathrm{Fe}]\times[\mathrm{HCN}]}{[\mathrm{FeHCN}]} = K,$$

or if n denotes the uninhibited portion of the enzyme reaction we have

$$\frac{n}{1-n}[\mathrm{HCN}] = K,$$

where K is the hydrogen cyanide concentration causing half inhibition of the reaction.

For example, we found that for the inhibition of the respiration of avian red blood cells‡

[HCN] *mole/litre*	*Residual respiration n*	*K mole/litre*
$0{\cdot}5\times10^{-4}$	0·37	$0{\cdot}29\times10^{-4}$
$1{\cdot}0\times10^{-4}$	0·19	$0{\cdot}23\times10^{-4}$
10×10^{-4}	0	..

† H. Wieland and A. Bertho, *Liebigs Annalen*, **467**, 95 (1928).
‡ Morizo Onaka, *Z. f. physiolog. Chem.* **70**, 433 (1911) [p. 438].

For the inhibition of the respiration of rat tissue, Howard L. Alt† found:

[HCN] mole/litre	*Respiration inhibition* $[(1-n)\times 100]$		
	Kidney	*Liver*	*Spleen*
4×10^{-4}	63%*	50%*	(91%)
20×10^{-4}	89%	87%	82%*
100×10^{-4}	96%	99%	95%

If the K values are calculated from the figures marked with an asterisk and these values are used for calculating respiration inhibition for the higher cyanide concentrations we obtain:

	Kidney $K = 2{\cdot}4\times10^{-4}$	*Liver* $K = 4\times10^{-4}$	*Spleen* $K = 4{\cdot}4\times10^{-4}$
Inhibition by [HCN] $= 20\times10^{-4}$	calc. 89% found 89%	calc. 82% found 87%	.. found 82%
Inhibition by [HCN] $= 100\times10^{-4}$	calc. 98% found 96%	calc. 96% found 99%	calc. 96% found 95%

It can be seen from these examples that the simple dissociation law agrees as well as can be expected from the error of the determination. The question as to how complete is the inhibition by cyanide is answered by the statement that it is as complete as is required by the dissociation law.

8. Sea urchins' eggs

For fertilized sea urchins' eggs I found that the concentration of hydrogen cyanide which produces half inhibition of the respiration is:‡ $0{\cdot}5\times10^{-5}$ mole/litre.

It struck me then, for the first time, how different was the oxidation inhibition by cyanide from the oxidation inhibition by narcotics. A fertilized egg whose respiration had been reduced to half by narcotics had completely lost its ability to divide. In hydrogen cyanide, however, the egg divided normally, only in general more slowly, as its respiration was inhibited.

† Howard L. Alt, *Bioch. Zeitschr.* **221**, 498 (1930).

‡ O. Warburg, *Z. f. physiolog. Chem.* **66**, 305 (1910), p. 325.

9. Red blood cells

The respiration of all types of red blood cells, with or without nuclei, is inhibited by hydrogen cyanide. The hydrogen cyanide concentration which brings about half inhibition is about 3×10^{-5} mole/litre.†

For the reason mentioned in section 6 of this chapter, the presence in the blood cells of methaemoglobin must be avoided.

The glycolysis of the red blood cells is also inhibited by cyanide, but only, however, by very much higher concentrations than is the case with the respiration. 10^{-2} N hydrogen cyanide reduces the glycolysis to the half value. The glycolysis of the red blood corpuscles is, therefore, about 300 times less sensitive than the respiration of the red blood cells. The respiration of the red blood cells can, therefore, be completely inhibited without any inhibition of the glycolysis. If the respiration is inhibited the glycolysis actually increases from the aerobic to the anaerobic value.

10. Yeast

Both the respiration and the fermentation of yeast are inhibited by cyanide. The half-value concentrations are:‡

For the inhibition of the respiration $0{\cdot}45 \times 10^{-5}$ mole/litre (Bakers' yeast).
For the inhibition of the fermentation $1{\cdot}0 \times 10^{-2}$ mole/litre (Bakers' or Brewers' yeast).

For yeast cells also the respiration—and the fermentation—inhibiting hydrogen cyanide concentrations lie far apart, so that in this case also, respiration and fermentation can be differentiated by cyanide. Again, when the respiration is inhibited the fermentation increases from the aerobic to the anaerobic value.§

On account of their high metabolic rate, the simple requirements for their culture, and the variability of their metabolic types, yeast cells are particularly suitable experimental material. Wild yeasts have a high respiration. The rate of respiration of

† Morizo Onaka, *Z. f. physiolog. Chem.* **70**, 433 (1911) [p. 438].
‡ O. Warburg, *Bioch. Zeitschr.* **165**, 196 (1925); E. Negelein, ibid. **165**, 203 (1925).
§ O. Meyerhof, ibid. **162**, 43 (1925).

cultured yeasts depends on the conditions of culture.† Brewers' yeast is unsuitable for respiration work on account of its small and irregular respiration.

11. Lactic acid bacilli

Aerobic lactic acid bacilli behave towards hydrogen cyanide in the same way as Brewers' yeast, i.e. the respiration and the fermentation are inhibited by cyanide, the former by very small, the latter by very much greater, concentrations. In this case also, respiration and fermentation can, therefore, be differentiated by hydrogen cyanide.

The anaerobic lactic acid bacilli are different. Their fermentation, like that of the aerobic type, is inhibited by cyanide, but the respiration behaves differently in accordance with the conditions of culture.

The respiration of the anaerobic lactic acid bacillus Delbrückii of the Kiel Meyerhof Institute is inhibited by cyanide. J. G. Davis‡ in the Meyerhof Institute found that the hydrogen cyanide concentration for half inhibition was about 5×10^{-4} mole/litre.

The respiration of the anaerobic lactic acid bacillus Delbrückii of the Berlin Fermentation Institute, according to Meyerhof and Finkle§ was not inhibited by cyanide.

The bacillus of the Fermentation Institute at Reading in England is a third anaerobic lactic acid bacillus whose metabolism has been studied. The fermentation of this bacillus behaves towards cyanide like that of the other lactic acid bacilli. In so far as the respiration is concerned, however, J. G. Davis‡ found that it does not possess any.

Davis's observation seems to me to offer the key to the problem of the respiration of lactic acid bacilli. Apparently when these are cultured anaerobically, they lose gradually the enzyme system of respiration. The Kiel bacillus still had the whole chain of respiratory enzymes with oxygen transporting

† O. Warburg, ibid. **189**, 350 (1927).
‡ J. G. Davis, ibid. **265**, 90 (1933); **267**, 357 (1933).
§ O. Meyerhof and P. Finkle, *Chemie der Zelle*, **12**, 157 (1925).

iron at the head. The Berlin bacillus had lost its oxygen transporting iron, but still retained the other respiratory enzymes, in this case with the yellow enzyme at the top, this being uninhibited by the cyanide. In the English bacillus the breakdown of the enzyme chain had advanced still farther, in that the yellow enzyme had also disappeared, so that the final result of anaerobic culturing had been fully realized.

It is well known that lactic acid bacilli have played a great part in the discussions about oxygen transporting iron.† It can be said to-day that it would have been more logical to study the exception until it had been fitted into the general principle rather than to dispute the general principle on account of the exception.

12. Hydrogen producing bacteria

M. Stephenson and L. H. Stickland‡ found that the decomposition of formic acid by *Bacillus coli*,

$$HCOOH = CO_2 + H_2,$$

is inhibited by hydrogen cyanide and, moreover, by concentrations 1,000 times less than is lactic acid fermentation or alcoholic fermentation. The hydrogen cyanide concentration which produced half inhibition of the formic acid fermentation was

$$1 \times 10^{-5} \text{ mole/litre.}$$

Another fermentation in which hydrogen is also produced, butyric acid fermentation,

$$C_6H_{12}O_6 = C_4H_8O_2 + 2CO_2 + 2H_2,$$

is particularly sensitive to cyanide. In this case we found§ that it is only a part of the butyric acid fermentation that is cyanide sensitive, not the breakdown of the sugar, but the reaction by which hydrogen is produced from the breakdown products. If very dilute hydrogen cyanide is added to the fermenting butyric acid bacteria, the hydrogen evolution ceases, but break-

† A. Bertho and H. Gluck, *Liebigs Ann.* **494**, 159 (1932); A. Bertho, *Ergebnisse der Enzymforschung*, **2**, 204 (1933).

‡ M. Stephenson and L. H. Stickland, *Bioch. Journ.* **26**, 712 (1932).

§ F. Kubowitz, *Bioch. Zeitschr.* **274**, 285 (1934).

down of the sugar continues, to give, not a butyric acid fermentation, but a lactic acid fermentation.

13. Chlorella

The green alga Chlorella has been very closely studied. Four metabolic processes have been tested in regard to their behaviour towards cyanide. These are: the autotrophic respiration, the heterotrophic respiration, carbon dioxide assimilation, and nitrate assimilation. The autotrophic respiration is that taking place when chlorella is cultured in solutions of inorganic substances. The heterotrophic respiration appears if the chlorella, cultured in an inorganic medium, is placed for 15 minutes in sugar solution and then brought back into the sugar-free salt solution. Under these conditions, as Emerson found, the respiration increases five-fold.

The hydrogen cyanide concentrations which produce half inhibition of the four metabolic processes are:

	[HCN] *mole/litre*
Autotrophic respiration† . . .	greater than 10^{-1}
Heterotrophic respiration‡ . .	1×10^{-4}
Carbon dioxide assimilation† . .	4×10^{-5}
Nitrate assimilation† . . .	$0{\cdot}8 \times 10^{-6}$

The autotrophic respiration of chlorella was the first cyanide resisting respiration to be found. L. Genevois§ tried to find out whether the cyanide resisting respiration was a property which differentiated plant cells from other cells, or green cells from those not green. He found that the respiration of germinating plants is very sensitive to cyanide and, moreover, that the respiration of many green autotrophic algae is also sensitive to cyanide. Autotrophic chlorella is, therefore, an exceptional case.

14. Respiration of heart muscle

Claude Bernard found that the respiration of heart muscle was inhibited by cyanide, and that the heart blood of cyanide-poisoned animals was still bright red a day after death.

† O. Warburg, ibid. **100**, 230 (1919); **166**, 386 (1925).
‡ R. Emerson, *Journ. General Physiology*, **8**, 469 (1927).
§ L. Genevois, *Bioch. Zeitschr.* **191**, 147 (1927); **186**, 461 (1927).

V. v. Weizsäcker examined the effect of cyanide on the isolated frog heart, whilst the heart was beating and working, that is, under conditions such that the heart muscle was intact. He found:†

[HCN] *mole/litre*	*Inhibition of respiration*
$0{\cdot}5 \times 10^{-4}$	29%
$2{\cdot}5 \times 10^{-4}$	70%
$5{\cdot}0 \times 10^{-4}$	95%

15. Respiration in solutions

In 1909 Batelli and Stern‡ showed that aqueous extracts of animal tissues take up oxygen and give off carbon dioxide. Since the respiration in the aqueous extracts was small in comparison with that of the intact tissue, Batelli and Stern called the former 'accessory respiration' in contrast to the main respiration of the intact cells. Buchner yeast press juice, and aqueous extracts of dried yeast also respire. Again, such respiration is smaller than that of the yeast cells.

Batelli and Stern found that the respiration in aqueous solution, in contrast to the cell respiration, is little inhibited by cyanide. Probably the respiration in solution is not at all inhibited by cyanide, when the solid cell particles are completely removed by high-speed centrifuging. The respiration of press juice, completely clarified by centrifuging is not only uninhibited by cyanide, but is even increased.

How is it possible to obtain from cells, the respiration of which is completely inhibited by cyanide, a respiration which is increased by cyanide?

The answer to this question lies in the enzyme chain, and could only be given when the yellow enzymes, which come into the chain reaction after the oxygen transporting iron, had been discovered.§ The accessory respiration of Batelli and Stern and the respiration in the clarified yeast juice are due to

† V. v. Weizsäcker, *Pflügers Archiv*, **147**, 135 (1912).

‡ Batelli and Stern, *Bioch. Zeitschr.* **21**, 487 (1909); *Ergebnisse der Physiologie*, **12**, 96 (1912).

§ O. Warburg and W. Christian, *Bioch. Zeitschr.* **254**, 438 (1932).

oxygen transport by the yellow enzymes which are separated from the insoluble iron system by the extraction of the cells. Since cyanide does not inhibit the yellow enzymes the respiration in solution is unaffected by cyanide.

The reason for hydrogen cyanide increasing the respiration in solution depends on hydrogen peroxide and catalase. In oxygen transport by the yellow enzymes, hydrogen peroxide is formed,† and this is decomposed by catalase with liberation of oxygen. Since the catalase is inhibited by cyanide, the formation of oxygen from hydrogen peroxide is also inhibited, so that, in the presence of enough cyanide to inhibit the catalase completely, the oxygen requirement is doubled.

This only applies if the oxygen transporting enzyme in the cell is an iron compound. When the oxygen is being transported by copper‡ as in the potato and in fungi, the respiration in aqueous extracts of the material is inhibited by hydrogen cyanide. The reason for this is that, unlike the oxygen transporting iron proteins, the oxygen transporting copper proteins are soluble in water.

16. Experiments of Dixon and Elliot

The experiments of Batelli and Stern were repeated and verified in 1939 by Dixon and Elliot. They made pulp preparations from heart, liver, kidney, and spleen, suspended these in phosphate buffer, and determined the oxygen uptake manometrically.

They found§ that the respiration was only inhibited to about 50 per cent. by cyanide. Apparently the respiration of finely divided tissue was made up by approximately equal amounts of the main respiration and the accessory respiration. In contrast, however, to Batelli and Stern, Dixon and Elliot concluded that the respiration of the intact cells could only be inhibited to the extent of 50 per cent. by cyanide.

Howard L. Alt‖ repeated the experiments of Dixon and

† Ibid. **266,** 377 (1933) [p. 395].
‡ F. Kubowitz, ibid. **292,** 221 (1937).
§ M. Dixon and K. A. C. Elliot, *Biochem. Journ.* **23,** 812 (1929).
‖ Howard L. Alt, *Bioch. Zeitschr.* **221,** 498 (1930).

Elliot with the modification that Ringer-bicarbonate instead of phosphate solution was used. Instead of having potassium hydroxide in the inner container of the manometer vessel, the method employing a pair of vessels was used, and the respiration determined under the physiological carbon dioxide pressure. Loss of cyanide by distillation into the inner container was thereby prevented.

Under these conditions the respiration of liver, kidney, and spleen was inhibited to the extent of 95 per cent. by N/100 hydrogen cyanide. This result is the same as that calculated from the dissociation law (section 7 of this chapter) using the value for the hydrogen cyanide concentration which produces half inhibition. One hundred per cent. inhibition would only be realized by infinitely greater cyanide concentration, just as it is possible to obtain 100 per cent. saturation of haemoglobin with oxygen only by using infinitely great concentrations. This is the answer to the illogical but often repeated question as to whether inhibition of respiration by cyanide can be complete.

The work of Alt had shown that the technique employing minced tissue, when rightly used, can be applied to experiments with sensitive animal tissues for which it was thought to be unsuited. However, it must not be thought that the manometric method for studying metabolism is the only one available in physiology. Dixon and Elliot should at least have made certain that their findings were correct by employing other methods, for example, the perfusion technique, before accepting results which disagreed with earlier ones.

For when Claude Bernard† found in 1857 that the blood from the heart of a cyanide-poisoned animal was still bright red 24 hours after death, it meant, taking into account the oxygen requirement of the heart and the oxygen capacity of the blood, that the respiration of the heart muscle had been completely inhibited. Also, since 1912 there had been on record the researches of Weizäcker‡ who found complete inhibition of the respiration of the frog heart poisoned by cyanide.

† Section 1 of this chapter.
‡ Section 14 of this chapter.

In spite of this, in 1929 the result obtained by Dixon and Elliot using minced heart muscle was accepted by many without question. For example Torsten Tunberg† wrote:

The view that the respiration enzyme is responsible for the whole aerobic respiration cannot be correct in view of the fact that cyanide inhibits in many cells only a part of the oxygen uptake. This observation had already been made in respect of certain cells, but Dixon and Elliot, who in 1929 examined the effect of varying concentrations of potassium cyanide on the oxygen uptake of different animal tissues, proved that on an average 40 per cent. of this uptake is unaffected by the cyanide.

It must not be forgotten that the inhibition of the respiration by cyanide was not compatible with the Wieland theory. Therefore, the cyanide inhibition had to be at least incomplete; at least a part of the respiration had to be saved for the theory. This was the reason for the remarkable literature on the theme: Is the inhibition of the respiration by cyanide complete?

17. Animal cells and tissue

The aerobic respiration and the anaerobic glycolysis of all animal cells and tissue which we have examined are inhibited by cyanide. The aerobic and the anaerobic inhibiting concentrations differ sufficiently to allow a separation of the one from the other. Under such conditions, in spite of the cells having been saturated with oxygen, the respiration increases from the aerobic to the anaerobic value.

Animal cells and tissue for which this increased respiration in the presence of cyanide has been determined include tumours,‡ embryos,‡ and red blood cells.§

18. Ethyl isocyanide

In contrast to hydrogen cyanide, its ethyl ester

$$C \leqq N—C_2H_5$$

does not inhibit either the aerobic respiration or the anaerobic

† Torsten Tunberg, *Oppenheimers Handbuch der Biochemie, Ergänzungsband*, p. 270 (Jena, 1930).

‡ O. Warburg, K. Posener, and E. Negelein, *Bioch. Zeitschr.* **152**, 309 (1924).

§ O. Warburg, F. Kubowitz, and W. Christian, ibid. **242**, 170 (1931).

glycolysis or the carbon dioxide assimilation, provided of course that the concentrations applied are within the limits of the narcotic concentrations. For example, we found:†

	Oxygen consumption per mg. dry weight per hour	
	In 10^{-3} *N Ethyl isocyanide c.mm.*	*Without addition c.mm.*
Liver . .	11·2	10·4
Kidney . .	25·7	23·7
Testicle . .	11·2	11·4
Embryo . .	13·6	13·6
Jensen Sarcoma	13·8	13·2

Although the aerobic respiration of these tissues is not inhibited by ethyl isocyanide, the fermentation increases—in the presence of oxygen—to the anaerobic value. The Pasteur effect is therefore completely inhibited by the cyanide ester. An explanation of this observation will perhaps come as a result of the recent work on zymohexase and its activation by iron salts.‡

The nitriles which are isomeric with the cyanide esters

$$N{\equiv}C{-}C_2H_5$$

have no specific action on metabolic processes, although statements to the contrary are to be found in the literature. They are actually indifferent narcotics.

19. Hydrogen sulphide

At one time, before it was known why cyanide inhibited chemical reactions in the cell, certain substances were regarded as important because they were comparable with cyanide as regards their biological activity. We found that hydrogen sulphide§ was such a substance, as the following table shows:

† O. Warburg, *Bioch. Zeitschr.* **172**, 432 (1926).
‡ Cf. Chapter XIX.
§ E. Negelein, *Bioch. Zeitschr.* **165**, 203 (1925).

	10^{-4} N H_2S	10^{-4} N HCN
Respiration of yeast cells	Strong inhibition	Strong inhibition
Fermentation of yeast cells	No inhibition	No inhibition
Carbon dioxide assimilation	Strong inhibition	Strong inhibition
Nitrate assimilation	Strong inhibition	Strong inhibition
Respiration of Chlorella (autotrophic)	No inhibition	No inhibition
Respiration of Chlorella (heterotrophic)†	Strong inhibition	Strong inhibition

In 1925 this parallelism between the two substances lent considerable support to the heavy-metal theory of respiration and served as an argument against other theories, for example, against the theory that the action of hydrogen cyanide was due to the formation of cyanhydrins.

20. Catalysis and anticatalysis

Since a small amount of cell substance can deal with large amounts of substrate, aerobic respiration, anaerobic respiration and carbon dioxide assimilation are catalytic processes, and since a small amount of cyanide will inhibit the metabolism of much substrate, cyanide inhibition is therefore an anticatalytic process.

If one knew the chemical nature of catalysts it would be easy to find anticatalysts on the basis of the latter being substances that react chemically with the catalysts. Vice versa, if the catalyst is unknown, it is possible to obtain information as to its nature from a knowledge of the chemical properties of the anticatalyst. The great importance of cyanide inhibition in enzyme chemistry thus becomes apparent. If our supposition were correct, namely, that cyanide inhibition is not a narcotic inhibition, but a chemical anticatalysis, then it would follow that the enzymes of aerobic respiration, anaerobic respiration, carbon dioxide and nitrate assimilation must have catalytically active groups which are chemically alike. This is the principle underlying our investigation of biochemical processes.

† R. Emerson, *Journ. General Physiology*, **8**, 469 (1927).

CHAPTER IV

IRON CATALYSIS AT SURFACES

BLOOD CHARCOAL

In 1921 I found that amino-acids adsorbed from aqueous solution on the surface of blood charcoal are oxidized at room temperature and neutral reaction by molecular oxygen. These oxidations, like cell respiration, are inhibited non-specifically by narcotics according to the homologous series rule, and specifically by cyanide. When one considers that at that time biocatalysis and its inhibition were still as mysterious as at the time of Mitscherlich and Claude Bernard, one can understand the trouble we have taken in our study of the charcoal model.

There are good models and bad models. When sugar is decomposed by heating with alkali, or carbon dioxide is decomposed by irradiation with short-wave ultra-violet light, we have bad respiration and assimilation models, because the conditions under which the corresponding biological processes take place are not adhered to. Who could doubt, however, that the assimilation problem would be solved if, in a model experiment, the decomposition of carbon dioxide were brought about by quanta of visible light?

1. Oxidation of amino-acids†

If the oxidation of an α-amino-acid, for example leucine, is allowed to go to completion on blood charcoal, then the oxygen uptake ceases when one molecule of carbon dioxide and ammonia are formed from each molecule of leucine according to the equation

$$\begin{array}{l} \mathrm{R} \\ | \\ \mathrm{CH \cdot NH_2} \\ | \\ \mathrm{COOH} \end{array} + \mathrm{O_2} = \begin{array}{l} \mathrm{R} \\ | \\ \mathrm{COOH} \end{array} + \mathrm{CO_2} + \mathrm{NH_3}.$$

Cystine requires more oxygen than corresponds to this

† *O. Warburg and E. Negelein, Bioch. Zeitschr.* **113**, *257 (1921);* **142**, *493 (1923);* **152**, *191 (1924).*

equation, because the sulphur is oxidized in the presence of blood charcoal to sulphuric acid.

2. Inhibition by narcotics†

If any narcotic, in sufficient concentration to reduce the 'respiration' of the charcoal to the half value, is added to a suspension of blood charcoal which has adsorbed 0·05 millimole of cystine per gram, then if the amount of cystine adsorbed is redetermined, 0·025 millimole only is found. The narcotics, therefore, displace the amino-acids from the surface of the charcoal, and since the inhibition of the oxidation and the degree of displacement correspond, the inhibition of the oxidation must have been caused by this displacement.

The fact that the oxidation of the amino-acids, which are quite stable in solution, comes to a stop if they are displaced from the charcoal is in itself informative. Narcotic inhibition of respiration—in this case the oxygen uptake of the charcoal—requires not merely a displacement, but the displacement of a substance unstable on the surface by one which is stable. This condition is realized by most narcotics. We have tried to answer the question as to what determines the displacing action of the narcotics, and have investigated for several of them how much must be adsorbed on the surface before a certain degree of displacement is reached. We found that the area which the narcotic takes up on the surface of the charcoal determines the displacement. It was immaterial whether the narcotic was urethane, a ketone, or a nitrile: the same amount of cystine was displaced from the charcoal when a certain fraction of its surface was covered by the narcotic. Narcotic inhibition of charcoal respiration is, therefore, a physical phenomenon. The rule for the homologous series can, therefore, be explained. In ascending a homologous series the adsorption constants and molecular volumes increase. Therefore, for the same concentrations of dissolved narcotics the area of the charcoal covered by the narcotic becomes progressively greater.

† *O. Warburg, ibid.* ***119***, *134 (1921).*

3. Cyanide inhibition†

Hydrogen cyanide is also adsorbed by charcoal, and amino-acids already adsorbed can be displaced by it. Hydrogen cyanide, however, is only weakly adsorbed, less so than acetamide and acetonitrile which are the weakest narcotics. If the acetamide and acetonitrile concentrations which produced half displacement of cystine were 0·2 molar, the hydrogen cyanide concentration producing an equal displacement would be 1/1 molar. When, therefore, Wieland‡ and Bertho§ ascribed the effects of hydrogen cyanide, which they considered to be acting as a narcotic, to its particularly great adsorption, they had reversed the property of the hydrogen cyanide.

If hydrogen cyanide were inhibiting charcoal respiration by displacement as in the case of narcotics, then for the cyanide we should also have

Degree of inhibition = Degree of displacement.

Also, there should be half inhibition of the cystine oxidation when the cyanide concentration in solution was 1/1 molar. Actually half inhibition of the oxidation is observed at a hydrogen cyanide concentration 2,500 times smaller, i.e. at $0{\cdot}4 \times 10^{-3}$ mole/litre. At this low cyanide concentration no trace of displacement of the amino-acid is detectable. Hydrogen cyanide, therefore, inhibits the charcoal respiration without displacing the amino-acid from the surface.

4. Active regions‖

In the case of cyanide inhibition, a determination was made of the amounts of amino-acids and cyanide simultaneously adsorbed on the charcoal surface. The arrangement of this important experiment was as follows:

To 50 c.c. of a solution, M/20 in respect of leucine and M/1000 in respect of cyanide, 2 g. of blood charcoal was added, and the mixture shaken for several minutes till equilibrium was estab-

† O. Warburg, *Bioch. Zeitschr.* **119**, 134 (1921); **136**, 266 (1923).
‡ H. Wieland, *Liebigs Ann.* **445**, 181 (1925).
§ A. Bertho, *Ergebnisse der Enzymforschung*, **2**, 204 (1933).
‖ O. Warburg, *Bioch. Zeitschr.* **136**, 266 (1923).

lished. The charcoal was centrifuged off, and the supernatant liquid titrated in the presence of formaldehyde to determine leucine, and with silver nitrate to give cyanide. We found:

	Concentration in the solution mole/litre	*Adsorbed mole/per g. charcoal*
Leucine . . .	$3{\cdot}6 \times 10^{-2}$	$0{\cdot}70 \times 10^{-3}$
Hydrogen cyanide .	$0{\cdot}08 \times 10^{-2}$	$0{\cdot}01 \times 10^{-3}$

Since, therefore, one molecule of cyanide completely inhibits the oxidation of 70 molecules of adsorbed leucine, there is no other explanation than that only a small part of the surface is catalytically active. Leucine adsorbed on the greater part of the charcoal surface is just as resistant to the oxygen as non-adsorbed leucine in solution.

Thus were the active regions of charcoal discovered. The area of these regions in our preparation amounted to about 0·5 sq. metres per g. of charcoal on a total adsorbing surface of 200 sq. metres.

Note. After the discovery of the active regions, the equation which we had found applicable to the narcotic inhibition of charcoal respiration:

$$\text{Degree of inhibition} = \text{Degree of displacement}$$

was no longer as self evident as it appeared at first. It is obvious from the equation that narcotics displace amino-acids from all regions of the charcoal, the active as well as the inactive, to the same degree.

5. Chemical composition of the active regions†

Technical blood charcoal is obtained by carbonizing blood in the presence of salts, and is a complicated mixture containing, besides the carbon, much ash, oxygen, sulphur, and nitrogen. In order to learn more about the active regions, we changed the method of preparation, and set ourselves the task of building up, step by step, charcoals with the catalytic properties of technical blood charcoal.

Whilst sugar charcoal is autoxidizable and, therefore, cannot

† O. Warburg and W. Brefeld, ibid. **145**, 461 (1924).

be used in catalytic experiments, it is possible to obtain a highly adsorbent non-autoxidizable charcoal by carbonizing sugar in the presence of silicates. This charcoal is completely inactive, and obviously corresponds to the catalytically inactive material in blood charcoal.

Activation of the silicate charcoal with simple iron salts is not possible no matter whether the iron salts are added before or after heating to redness. If, however, a small quantity of haemin is added to the mixture of silicate and sugar before heating, a charcoal is obtained having the catalytic properties of the blood charcoal which had been inactivated by small amounts of cyanide.

In general, it appeared that nitrogen and iron must act in combination if the active cyanide-sensitive charcoals with the properties of blood charcoal were to be obtained. We concluded, therefore, that iron combined with nitrogen produced the chemically active part of the active regions of blood charcoal. The most active charcoals which we have obtained— charcoals which, however, differ from blood charcoal in some respects— were prepared from pure haemin.

It seemed to us important to be able to produce the iron-nitrogen combination step by step, so carbonization of porphyrin and then heating it to redness with iron salts should have been the next experiment. It is, however, difficult to obtain the necessary iron-free porphyrin. We found the aniline dyestuff Bismarck-brown more suitable as the starting material, as we could obtain this in a practically iron-free condition (iron content 0·001 per cent.).

Charcoal prepared from this Bismarck-brown contained 10 per cent. nitrogen and 0·01 per cent. iron. It was only feebly active catalytically. If, however, the iron content was increased by heating to redness with iron salts, the catalytic activity increased 10 to 20 times. On the addition of cyanide the whole effect of the iron disappeared. On account of its great importance in the study of the respiration problem, this experiment is graphically represented in Fig. 2.

To summarize, the result of the study of the charcoal model

containing iron bound to nitrogen was that this combination produced a biological oxidation like that of the oxidative deamination of amino-acids, an oxidation which is reversibly inhibited by narcotics and irreversibly and specifically inhibi-

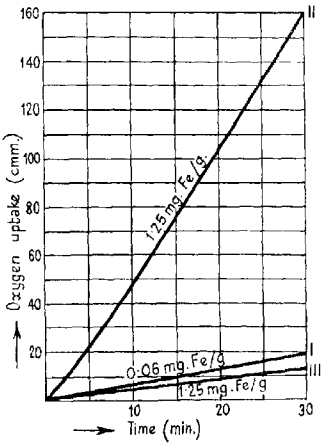


FIG. 2. In each case 20 mg. charcoal + 10 c.c. solution. I. Carbon, not activated, in pure leucine solution (N/20). II. Carbon, activated with iron, in pure leucine solution (N/20). III. Carbon activated with iron, in N/20 leucine and N/1000 cyanide.

ted by cyanide. Who could believe that this was only by chance in agreement with the behaviour of cell respiration?

E. K. Rideal and W. M. Wright† repeated and verified our work on the active regions of blood charcoal in 1925 and 1926. They found that blood charcoal consists of active and inactive regions and that the active regions contain nitrogen and iron. Rideal and Wright in my opinion lay too much stress on the question as to whether the inactive regions are completely inactive or only 800 times less active than the active regions. From an analytical point of view this question can scarcely be answered experimentally.

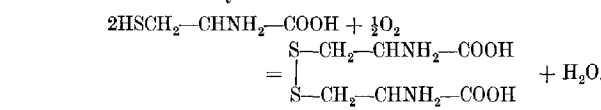
† E. K. Rideal and W. M. Wright, *Journ. Chem. Soc. London*, **127**, 1347 (1925); **129**, 1813 (1926); **129**, 3182 (1926).

CHAPTER V

IRON CATALYSIS IN SOLUTION

WHILST all other protein hydrolysis products are stable to molecular oxygen at physiological temperature and pH, E. Baumann† found that cysteine

is oxidized to cystine when it stands in air.



Baumann found that the oxidation of cysteine is accelerated by iron salts. In this reaction the solution becomes coloured, a phenomenon which explains the mechanism of the action of the iron.

If a little ferric salt is added to a neutral solution of cysteine, a violet colour develops. This disappears on standing, but reappears on shaking the solution with air, and the process may be continued till the whole of the cysteine has been oxidized to cystine. The iron thus transports oxygen by means of the valency change ferrous ⇌ ferric. The violet-coloured ferric cystine oxidizes excess cysteine to cystine, and the colourless ferrous cysteine is re-oxidized by molecular oxygen to the ferric compound.

In 1909 Matthews and Walker‡ observed that this action of the iron is inhibited by cyanide. Since hydrogen cyanide forms with iron a more stable complex than does cysteine, it therefore removes the iron from the cysteine and brings the catalysis to an end. Actually the colour development discovered by Baumann is no longer observed when the cysteine solution contains cyanide.

There is no clearer example of catalysis and anticatalysis

† E. Baumann, *Z. f. physiologische Chem.* **8**, 299 (1883–4).

‡ A. P. Matthews and S. Walker, *Journ. Biol. Chem.* **6**, 29 (1909).

than this. One can see how the complex autoxidizable iron compound is formed, how the complex ferric iron in it reacts by changing its valency, and how cyanide decomposes the complex iron compound. The finding of Matthews and Walker, however, that both the autoxidation and the iron accelerated oxidation of cysteine are inhibited by cyanide was not understood.

Matthews and Walker refused to accept the logical view that the 'autoxidation' of cysteine might be a catalytic oxidation brought about by impurities of heavy metals in solution, because after careful recrystallization the autoxidation did not disappear but remained quite unchanged.

1. The active molecules

Why should traces of cyanide inhibit the autoxidation of cysteine? If the cyanide did not react with the cysteine then it could not inhibit, and if it did react, it would react stoichiometrically, and at most prevent only a small part of the cysteine from reacting with oxygen.

Matthews and Walker gave the following explanation: In a cysteine solution, only a small fraction of the molecules present at a given time are reactive. If a solution of hydrogen cyanide is added, then the hydrogen cyanide reacts with the active molecules and protects these from the oxygen. If the active cysteine which is combined with the hydrogen cyanide becomes later inactive, the hydrogen cyanide dissociates, and is ready to react as new active cysteine molecules are formed. Thus a small amount of cyanide can prevent a large amount of cysteine from reacting with oxygen.

This theory has also been used to account for other cases of cyanide inhibition. Thunberg† explained the cyanide inhibition of cell respiration on the assumption that hydrogen cyanide combined with the fraction of the molecular oxygen which was at the moment active, and prevented it, therefore, from oxidizing the biological substrates. Wieland‡ also explained in a corresponding manner the oxidation of iodic acid and its

† T. Thunberg, *Skandinav. Archiv f. Physiologie*, **35**, 163 (1918).
‡ H. Wieland and F. G. Fischer, *Chem. Berichte*, **59**, 1171 (1926).

inhibition by cyanide. In this case he believed that traces of cyanide reacted with the iodic acid molecules active at any particular time, and thus a little cyanide was able to inactivate greater amounts of iodic acid. All these theories have proved to be false. When hydrogen cyanide inhibits oxidation, it reacts with neither the cysteine, nor the molecular oxygen, nor the iodic acid.†

2. Preliminary experiments

The problem of the autoxidation of cysteine as set out above was not only interesting in itself, but was of general importance, because here we had a biological substrate reacting directly with molecular oxygen and making superfluous, at least in this case, oxygen transporting iron. And if such a direct and non-catalytic oxidation were inhibited by non-stoichiometric amounts of hydrogen cyanide, then the cyanide inhibition could not be an anticatalysis, and our method of attack on the problem of cell respiration would be wrong in principle. We have, therefore, during the years in which the heavy metal theory of respiration was developing, busied ourselves with a study of the autoxidation of cysteine. We started with the conviction that the cysteine or the solution in which the autoxidation was being studied had been contaminated with heavy metal salts:

Bei der Darstellung des Cysteins — Reduktion des Cystins mit Zinn und Salzsäure — ist eine Verunreinigung mit Eisen schwer zu vermeiden. Es ist uns nicht gelungen, eisenfreie Cysteinpräparate zu gewinnen. Wir nehmen deshalb zunächst an, dass auch in diesem Fall die Blausäurewirkung auf der Bindung des Eisens oder eines andern Schwermetalls beruft.

This observation taken from a publication in 1921‡ started attempts in many laboratories to purify cysteine with a view to either proving or disproving the iron theory of cell respiration.

3. Experiments of Abderhalden and Wertheimer

Abderhalden and Wertheimer§ purified cysteine using all

† O. Warburg, *Bioch. Zeitschr.* **174**, 497 (1926).

‡ O. Warburg, ibid. **119**, 134 (1921) [p. 152].

§ E. Abderhalden and E. Wertheimer, *Pflügers Archiv*, **197**, 131 (1922); **198**, 122 (1923).

methods available. They were able to obtain preparations which gave no red colour with thiocyanate, but the cysteine remained autoxidizable:

Aus allen unsern Versuchen geht hervor, dass auch ohne eine Spur Eisen die Oxydation des Cysteins erfolgt. Daraus ergibt sich, dass die Ansicht, die Otto Warburg ganz allgemein vertritt, in diesem Fall keine Geltung haben kann.

The cyanides probably act by uniting with the sulphur group of the cysteine in the position where the oxygen ordinarily unites, and thus check the oxidation.

This was the opinion of Matthews and Walker in 1909.

4. Experiments of Dixon and Tunicliffe

Dixon and Tunicliffe† came to a different conclusion. They examined the rate of the cysteine oxidation and believed that they found that the velocity of the oxidation increased with time. They concluded that it was the oxidation product of cysteine, cystine, which brought about the oxidation by some intermediate reaction. Actually, they found that cysteine was oxidized more quickly if cystine had been previously added to the solution. This was a very improbable result. Why should cystine transport molecular oxygen, and how could hydrogen cyanide react with it?

Probably in Dixon's experiments the velocity of oxidation increased with time because heavy metal salts were dissolved from the walls of the containing vessel, and the addition of cystine probably promoted the reaction owing to its being contaminated with heavy metal salts.

5. Pyrophosphate

Accepting the view that the autoxidation of cysteine was really an example of heavy metal catalysis, it was difficult to see why only cyanide and not other complex-forming substances should inhibit the oxidation. In testing several complex-forming substances we found that pyrophosphate inhibited the

† M. Dixon and Tunicliffe, *Proc. Royal Soc. London*, B **94**, 266 (1923).

reaction just as well as cyanide,† whilst orthophosphate, which does not form complexes, was without effect.

This important experiment which settled in principle the problems of the autoxidation of cysteine is illustrated in Fig. 3.

At any rate, from this time on, all the theories according to which the cyanide reacted with the cysteine sulphur were

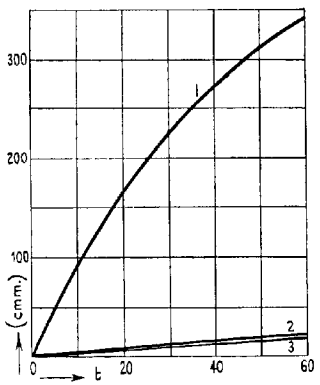


FIG. 3. Curve 1: Cysteine in water
Curve 2: Cysteine in pyrophosphate } pH 9·24.
Curve 3: Cysteine in cyanide

refuted, even if one did not admit the stoichiometric view and believed in activated molecules. For how could the pyrophosphate react with cysteine sulphur?

Pyrophosphate has often been used in biochemistry for a similar purpose, but not always in an appropriate manner. Like every complex-forming substance, pyrophosphate can inhibit a heavy metal catalysis only when the phosphate complex is more stable than the catalytically active complex. It must also be borne in mind that heavy metal pyrophosphate compounds can act as oxygen transporting catalysts, e.g. iron pyrophosphate transports oxygen to carbohydrates,‡ and again

† O. Warburg and S. Sakuma, *Pflügers Archiv*, **209**, 203 (1923); S. Sakuma, *Bioch. Zeitschr.* **142**, 168 (1923).

‡ H. A. Spoehr, *Journ. Amer. Chem. Soc.* **46**, 1494 (1924).

copper pyrophosphate† transports oxygen to cysteine. Pyrophosphate diffuses only slowly, if at all, into the living cell, so that it cannot be used for the inhibition of metal catalysts *in vivo*.

6. Purification of cysteine

In order to substantiate our belief that pure cysteine is not autoxidizable, we attempted its purification. It is now just as easy to obtain analytically pure cysteine as it was difficult at first to remove the traces of heavy metal salts which were retained from its preparation.‡

We succeeded in finding a simple method.§ After reducing the cysteine with tin and hydrochloric acid we crystallized the cysteine hydrochloride from concentrated hydrochloric acid solution and then extracted the crystals with acetone till the extract was colourless. Cysteine hydrochloride is sparingly soluble in acetone, but the associated metal salt impurity, iron chloride, is readily soluble.

The cysteine thus obtained was not completely iron free, but it was pure enough for our purpose.

7. Buffer solutions and vessels

Neither Matthews and Walker, nor Abderhalden and Wertheimer, nor Dixon and Tunicliffe had envisaged the possibility that heavy metal salts might be present in the buffer solutions, and that the glassware might give up heavy metal salts to the solutions, particularly to those containing a substance like cysteine which so readily forms complexes. Of what use was such pure cysteine if it were dissolved in impure buffers and shaken up in vessels from the walls of which heavy metal salts were being dissolved?

We therefore carried out the oxidation of cysteine in quartz vessels, and we purified our buffer solutions, so that we could use as a test of purity the rate of cysteine oxidation in accordance with our above-mentioned convictions. In this way it was soon

† O. Warburg, *Bioch. Zeitschr.* **187**, 255 (1927).
‡ S. Sakuma, ibid. **142**, 68 (1923).
§ O. Warburg, ibid. **187**, 255 (1927).

shown that the rate of oxidation decreased as the methods for the removal of heavy metal salts were improved.

The thiocyanate reaction, the purity test used by Matthews and Walker and Abderhalden and Wertheimer, was unsuitable for the following reasons:

1. The thiocyanate reaction indicates only ferric iron, whereas ferrous iron must also be removed.
2. Iron was not the only metal to be removed. Removal of copper and manganese, which are likewise catalysts for the oxidation, was also necessary.
3. The objection to all stoichiometric tests was that their sensitivity was not great enough. In stoichiometric reactions the heavy metal reacts only once; in catalysis, however, it may possibly react over and over again.

8. Results

If the quotient

$$Q_{O_2} = \text{c.mm. } O_2 \text{ used/mg. cysteine/hour},$$

then for the purest preparations under the same conditions (20°, pH 7·4, air):

	Q_{O_2}
Preparation of Matthews and Walker . .	4·2
„ Dixon and Tunicliffe . .	12·6
„ Warburg and Sakuma . .	0·048

By our method of purification the rate of cysteine oxidation had therefore been reduced to 1/90 of the value of Matthews and Walker and to 1/260 of the value of Dixon and Tunicliffe. If we added to our pure cysteine solution iron, copper, or manganese salts, 1 mg. of the metal increased the oxygen uptake by about 100,000 c.mm. of oxygen per hour. Using this value and that for Q_{O_2} obtained from the rate of oxidation of our purified cysteine, we could calculate how much heavy metal was contained in our purest cysteine preparation on the assumption that metal-free cysteine is not oxidized. In this way we *found that per mg. cysteine*

$$0{\cdot}048/100{,}000 = 4{\cdot}8 \times 10^{-7} \text{ mg.}$$

of iron, copper, or manganese was present.

No stoichiometric reaction could show the presence of such quantities, and to eliminate amounts of this order in the solutions and vessels is, to say the least of it, very difficult. Whether pure cysteine is not oxidized at all or very much more slowly than one thought, is immaterial. The point is that the oxidation of cysteine which is inhibited by cyanide is not an autoxidation, but an example of oxygen transport by a heavy metal.

9. Determination of iron, copper, and manganese†

We have based a method for the detection and determination of iron, copper, and manganese on the catalytic oxidation of cysteine. Into the main vessel of a manometric apparatus are introduced pure cysteine and purified buffer solution; the side tube holds the solution to be investigated. The experiment is carried out in oxygen, not air as specified in the published method. The temperature of the thermostat is 20°. Before the contents of the side tube are added the rate of oxidation is first measured. This should be small if the method is to be sensitive. The solution containing the metal is then added from the side tube, and the increased rate of oxidation determined. If the apparatus has been calibrated with known amounts of heavy metal the amount of heavy metal added can be calculated from the increase in the oxidation rate. It is possible, moreover, by changing the buffer solution, to distinguish between copper, iron, and manganese. In borate buffer all three transport oxygen. In pyrophosphate solution, however, the catalysis with iron and manganese is inhibited, whilst the copper catalysis takes place even more vigorously than in borate. In calibrating our apparatus we found the following increased rates of oxidation produced by 1 γ of the metal in each case:

	c.mm. O_2/γ heavy metal/hour
Copper in pyrophosphate, pH 7·6 . .	1000
Iron in borate, pH 9·3	100
Manganese in borate, pH 9·3 . . .	*100*

The sensitivity of the method depends on the purity of the

† O. Warburg, *Bioch. Zeitschr.* **187**, 255 (1927).

cysteine, the buffer solution, and the vessels. If these were quite pure the method would be extremely sensitive. Without addition of metal the rate of oxidation would be nil; after addition of the metal it would only be necessary to wait till the volume was sufficiently great before taking the reading.

This ideal is not attainable, but it is easy to reach a state of purity such that 0·05 γ of copper can be detected and determined with an accuracy of about 5 per cent. The increase in the rate of oxidation for this amount of copper amounts to 50 c.mm. oxygen per hour calculated on the above calibration values.

Using this method we have discovered, for example, that phenol-oxidase is a copper protein. In this case it was not necessary to ash or hydrolyse the enzyme. The unhydrolysed enzyme was added from the side tube into the main vessel where it was split by pyrophosphate into protein and copper salt.

10. Glutathione†

In 1921 when the discussion over the autoxidation of cysteine started, F. G. Hopkins isolated from yeast cells glutathione, a tripeptide composed of glycine, glutamic acid, and cysteine. This substance has been found in all cells examined, and is apparently an integral part of living cells. Like cysteine, glutathione is oxidized at room temperature and at neutral reaction by molecular oxygen, and the disulphide corresponding to that of cysteine is formed. According to Hopkins there are present in cells substances which reduce the oxidized glutathione to the HS— compound. This observation indicates that glutathione behaves as an oxidation catalyst. The problem of the oxidation of glutathione by molecular oxygen was therefore of importance in connexion with respiration. Does the molecular oxygen react directly with the HS— group of glutathione, or does this oxidation also involve a heavy metal oxygen transfer?

D. C. Harrison‡ answered this question. He found that the oxidation of glutathione was in all respects similar to that of

† F. G. Hopkins, *Biochem. Journ.* **15**, 286 (1921); **19**, 787 (1925); *Journ. Biol. Chem.* **84**, 269 (1929). Hunter and Eagles, ibid. **72**, 147 (1927).

‡ D. C. Harrison, *Biochem. Journ.* **18**, 1009 (1924).

cysteine. The oxidation is brought about by heavy metal oxygen transfer. Cyanide inhibits the reaction by combining with the catalytically active heavy metal. The oxygen transfer by iron can also be observed in this case. Ferric glutathione formed by the addition of ferric salts has a violet colour. On standing, the colour disappears, and on shaking the solution with air it returns. If cyanide is added there is no colour change.

Doubt was thrown on this clear-cut result by Meldrum and Dixon† in 1930. They asserted that quite pure glutathione was not oxidized by molecular oxygen, even in the presence of iron salts, whilst quite pure cysteine with the same amount of iron was oxidized. They concluded from their observations that in the case of cysteine the heavy metal and cysteine were sufficient, but in the case of glutathione a third substance was necessary to enable the molecular oxygen to react. Meldrum and Dixon, however, could not put forward any views as to the nature of this third substance.

Meldrum and Dixon were misled, in that the complex iron compound of cysteine is much more stable than that of glutathione.‡ If it is required to obtain the same rate of oxidation in solutions of cysteine and glutathione respectively, containing the same amount of iron, the concentration of glutathione must be much greater than that of cysteine. For similar reasons H. Hartmann§ was led to believe that ferrous glutathione, in contrast to ferrous cysteine, does not react with carbon monoxide. Actually, after adding ferrous salts to glutathione solutions, carbon monoxide is only taken up if the glutathione concentration is very high.

11. Hydrogen sulphide and sulphides‖

Since cysteine and glutathione are derivatives of hydrogen sulphide, it seemed worth investigating whether pure hydrogen sulphide was autoxidizable.

When solutions of hydrogen sulphide, for which precautions

† N. U. Meldrum and M. Dixon, *Biochem. Journ.* **24**, 472 (1930).
‡ F. Kubowitz, *Bioch. Zeitschr.* **282**, 277 (1935).
§ H. Hartmann, ibid. **223**, 489 (1930).
‖ H. A. Krebs, ibid. **204**, 343 (1929).

had been taken to avoid the presence of heavy metal salts, were shaken with air at 20°, no oxygen uptake was observed by Krebs using the manometric technique. In view of the activity of the metal, this meant that less than 1/50,000 part of the hydrogen sulphide present was reacting per hour with oxygen.

When heavy metal salts were added the hydrogen sulphide was oxidized in air, nickel salts being particularly active. In strong hydrochloric acid solution iron and manganese were the most active metals.

Metal sulphides were more sensitive to the addition of heavy metal salts than the free hydrogen sulphide. Krebs found the following activities using half molar sodium sulphide solution at 20°.

	c.mm. O_2/mg. metal/hour
Manganese	$96{\cdot}0 \times 10^6$
Nickel	$6{\cdot}6 \times 10^6$
Cobalt	$0{\cdot}96 \times 10^6$
Iron	0·082
Copper	0·071

It can be seen, therefore, that a sodium sulphide solution containing 0·001 γ manganese required 100 c.mm. of oxygen per hour. Since such small amounts of metal, which are not detectable by stoichiometric test, cannot be excluded from solutions and apparatus, it must be assumed that the oxidation of sulphide solutions takes place by oxygen transfer by heavy metal salts.

12. Carbohydrates†

Another example of apparent autoxidation is that of carbohydrates in hydrochloric acid solution which we discovered in 1924. Although such oxidations probably do not take place in the living cell, they have been of importance in the respiration problem. More careful study showed that these apparent autoxidations were really examples of heavy metal catalysis. Like the oxidations of cysteine and glutathione, they were also inhibited by cyanide and pyrophosphate.

† O. Warburg and M. Yabusoe, *Bioch. Zeitschr.* **146**, 380 (1924); O. Meyerhof and K. Matsuoka, ibid. **150**, 1 (1924); F. Wind, ibid. **159**, 58 (1925); H. A. Krebs, ibid. **180**, 377 (1926).

13. Antioxygens

When aldehydes are shaken with air at room temperature, oxygen is absorbed. Benzaldehyde, for example, gives perbenzoic acid:

$$C_6H_5CHO + O_2 = C_6H_5COOOH.$$

Moureu and Dufraisse† found that this oxidation is inhibited by small amounts of various substances which they called antioxygens. Hydroquinone, for example, is an antioxygen, but not hydrogen cyanide. Wieland‡ expressed the opinion that the biological action of hydrogen cyanide and the action of these antioxygens on aldehydes were related phenomena.

Die antikatalytische Wirkung der Blausäure ist zweifellos allgemeinerer Art und kann nicht allein durch die Hypothese von der Erzeugung unwirksamer Metallcyanidverbindungen erklärt werden. Wir haben keine durchgreifend gültige Erklärung für sie, ebenso wie wir die seltsame Beobachtung von Moureu, nach der Autoxydationen durch minimale Spuren von Phenolen gehemmt werden, nicht erklären können.

Actually these two phenomena have nothing to do with one another. According to Orland M. Reiff,§ it appears that the oxidation of aldehydes takes place in a thin aqueous film on the walls of the vessel, and that the antioxygens cause inhibition by breaking up this film. To make biological comparisons, the antioxygens are to be compared with narcotics and not with hydrogen cyanide.

† Moureu and Dufraisse, *Compt. Rend.* **174**, 258 (1922); **175**, 127 (1922).

‡ H. Wieland in Oppenheimer's *Handbuch der Biochemie*, 2nd ed., **2**, 252 (1923).

§ Orland M. Reiff, *Journ. Amer. Chem. Soc.* **48**, 2893 (1926).

CHAPTER VI

IRON, THE OXYGEN TRANSPORTING CONSTITUENT OF THE RESPIRATION ENZYME

1. Historical

In Liebig's *Tierchemie* published in 1843 there is a chapter entitled 'Theorie der Respiration' in which combustion in the animal body is connected with the presence of iron. Liebig's iron, however, is not the iron of the tissue cells, but the haemoglobin iron of the red blood cells. Liebig believed that the combustion took place in blood, where the haemoglobin iron oxidized the biological substrates. 'Die Blutkörperchen enthalten eine Eisenverbindung, kein anderer Bestandteil der lebendigen Körperteile enthält Eisen.' If Liebig were right, the combustion would be of no value to the tissue cells since the energy of combustion can only be changed into work at the point where it is liberated. Liebig soon realized this and omitted the theory of respiration from the next edition of his *Tierchemie*. But the confusion surrounding oxygen transport has continued, and has not even to-day disappeared from chemical literature. Leaving aside all this confusion between oxygen transport and combustion, and between haemoglobin and catalytically active cell iron, there remains one historical work of note, that of Spitzer† in 1897. In this work the opinion was expressed that oxygen transfer was associated with iron in the cells, and an attempt made to prove this experimentally. Spitzer minced up tissue, precipitated it with acetic acid, and found that the precipitate promoted oxidations when in contact with air. For example, the oxidation of salicylaldehyde to salicylic acid, and that of the Nadi mixture to indophenol blue were brought about. Spitzer also found that the precipitate contained iron, and he expressed the view that the iron content and the oxidizing action were related.

Experimentally, the work of Spitzer was unsatisfactory, since the main bulk of the precipitate was inactive cell substance,

† W. Spitzer, *Pflügers Archiv*, **67**, 615 (1897).

and any decision as to whether the iron was associated with the inactive or the catalytically active part was impossible to make. Actually this iron was really haemoglobin iron. Nevertheless, Spitzer was right, because his precipitate did contain some oxygen transporting iron protein and cytochrome, i.e. the iron system of aerobic cells.

On account of the imperfect experimental proof, Spitzer's views attracted little attention. It happened that Röhmann,† under whose direction Spitzer's work had arisen, recalled in the year 1912 all the essentials of it. The year 1912 saw the birth of the Wieland theory and Röhmann's recapitulation was really published as a result of this. From then on, however, cell respiration was regarded as a direct autoxidation of biological substrates, oxygen transport by metals being superfluous.

A publication‡ on the part played by iron in the respiration of sea urchin's eggs which appeared in 1914 did nothing at first to alter the state of affairs at the time. Then the researches on iron catalysis at surfaces and in solutions, which have been dealt with in the previous chapter, caused some doubt as to whether science had followed the right road. This was roughly the position when, in 1924, I put forward a theory which was perhaps premature, but which future work has proved to be correct.

2. The theory§

Molecular oxygen which is used up by the respiration of aerobic cells never reacts directly with the biological substrates, but always and exclusively with divalent iron combined in a complex. Iron of a higher valency is thereby formed, and this is reduced back again to the divalent state by organic substances. Thus, in so far as the iron is concerned, the original state is regained. We therefore have a valency change in a complex iron compound by which the oxygen for the cell respiration is

† F. Röhmann and T. Shmamine, *Bioch. Zeitschr.* **42**, 235 (1912).
‡ O. Warburg, *Zeitschr. f. physiologische Chem.* **92**, 231 (1914).
§ O. Warburg, *Bioch. Zeitschr.* **152**, 479 (1924); *Chemische Berichte*, **58**, 1001 (1925).

transported, and in this sense iron is the oxygen transporting part† of the respiration enzyme.

In order to avoid any misunderstanding as to what is meant by the expression oxygen transport, I have graphically illus-

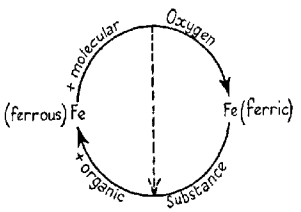


FIG. 4.

trated the theory, the valency changes of the iron being made clear by a cycle.

The arrow in the diagram connects the molecular oxygen with the organic substance. It is shown as a dotted line in order to indicate that reactions in the direction of the arrow do not take place in cell respiration.

3. Range of application of the theory

It has been said that the greater the value of a theory, the more specialized it is, and the more general is its application. The theory of oxygen transport by the valency change of an iron complex compound was very specialized. Its application was, as the future has shown, very general, since the reaction of divalent iron is really that reaction by which molecular oxygen most often reacts in biochemistry. An exception to this, which the theory of 1924 admitted, was the substitution of other heavy metals for the iron. 'Es mag Ausnahmen geben — man denke an das Kupfer der Oktopoden oder an Henzes Entdeckung des Vanadiums im Blut der Aszidien — aber derartige Fälle gehören nicht in eine allgemeine Theorie der Atmung.'

Thirteen years later we discovered that, in the respiration of

† 'Iron, the oxygen transporting part', could be more correctly written as 'iron, the autoxidizable part of the respiration enzyme'. [Compare Chapter XVI.]

the potato and of several fungi, the oxygen is transported, not by iron, but by copper.

The yellow enzymes which could have been used to attack the theory have, as was shown soon after their discovery, detracted nothing from the truth and applicability of the iron theory. The yellow enzymes transport oxygen in cell extracts and in such anaerobic cells as have a degenerating oxygen transporting system. In the aerobic cells, however, the yellow enzymes are intermediate members of the enzyme chain at the head of which stands oxygen transporting iron.

4. The iron compound

There are three types of substances mentioned in the theory, molecular oxygen, the iron compound and the organic substance.

As regards the iron compound, it was only stated in the theory that this was a complex. There are several reasons for this assumption. The ferrous ion is not autoxidizable, and cannot therefore transport oxygen. Ferrous hydroxide is, however, autoxidizable, but it combines so firmly with cyanide that the cyanide inhibition of respiration could not be reversible if this compound were involved.

In spite of our experiments with the blood charcoal no mention was made of haematin in the theory of 1924. Meyerhof and D. C. Harrison, however, believed our experiments sufficiently conclusive for their assertion that the oxygen transporting complex was a haematin derivative.

'We may imagine the structural surfaces of the cell to be like the charcoal surface, a mosaic of fields with and without iron, iron in definite combination similar to haematin.'†

'It is probable that oxidations in the tissue may be catalysed by organic compounds allied to haematin.'‡

If this were right then all aerobic cells would contain haematins. Actually the haematin of yeast was discovered by Hans Fischer and Hilger§ in 1924.

† O. Meyerhof, *Chemical Dynamics of Life Phenomena*, p. 22 (Philadelphia, 1924).

‡ D. C. Harrison, *Biochem. Journ.* **18**, 1009 (1924).

§ H. Fischer and J. Hilger, *Z. f. physiologische Chem.* **138**, 288 (1924).

5. The organic substance

In the graphic representation of the theory the 'organic substance' follows after the higher valency iron. The intention was to leave the relationship between these two as indefinite as possible since the course of the reaction of the ferric iron was really more problematical than was the oxidation of the ferrous iron. If the question were asked to-day, which substance reduces the iron of the oxygen transporting enzyme, the answer would be ferrous cytochrome. If, however, one includes the cytochrome iron with the oxygen transporting iron, then the course of the reduction of the iron is still a problem not completely solved. According to the work of A. v. Szent-Györgyi,† it is succinic acid in muscle which reduces the iron.

The organic substance mentioned in our theory of 1924 was immediately changed by others into 'activated substrate', so that the iron theory and that of Wieland could be combined. Thus the original iron theory was falsified, and the incorrect theory of Wieland was not made any less incorrect. The substrates, whether activated or unactivated, do not reduce the iron, but reduce the alloxazine—or the nicotinamide– conjugated proteins, about which nothing was known in 1924.

The indefinite expression 'organic substance' was therefore appropriate in 1924. Anyone taking exception to this demanded too much of the theory. The limiting of the theory to 'iron, the oxygen transporting part of the respiratory enzyme' was no defect in 1924, but rather an asset.

6. Hydrogen cyanide

The fact that cyanide combines with the oxygen transporting iron to give an inactive complex was no consequence of the theory, quite the reverse. From a knowledge of the chemical properties of the anticatalyst, the chemical nature of the catalyst was recognized.

As a result of the theory of 1924, the question was often asked whether the cyanide combined with the divalent or the trivalent iron, or both. This question was answered in 1927 by deter-

† A. v. Szent-Györgyi *et al.*, *Z. f. physiologische Chem.* **236**, 1 (1935).

mining the cyanide inhibition at different oxygen tensions.† The cyanide combines with the trivalent iron. We found that the cyanide inhibition of the respiration is independent of the oxygen pressure.

If the cyanide had reacted with the divalent iron, the molecular oxygen and the cyanide would have competed for the iron, and the inhibition of the respiration would have become smaller with increasing oxygen pressure.

7. Inhibition by narcotics

Oxygen transport by iron has in the theory been designated 'catalysis at surfaces', because narcotics inhibit cell respiration according to the same law as applies to the respiration of charcoal.

Kurt H. Meyer‡ opposed this on the grounds that it was mere analogy, and that decisive biological proof was not available. He was quite right. On the other hand he was unable to suggest a single model experiment or analogy for his own view that narcotic inhibition of biochemical reactions is brought about by solution of the narcotics in the fatty constituents of the cells. By this I mean that he could not put forward any chemical reactions which were inhibited by narcotics in accordance with his theory. On the contrary, Kurt Meyer's view has become less probable as our knowledge of the chemistry of enzymes has developed. How could the chemical reactions of conjugated proteins be inhibited by the solution of chemically indifferent substances like narcotics in the fatty constituents of the cell? If we take the opposite view that chemical reactions which are inhibited by narcotics according to the homologous series rule are invariably surface reactions, then the question might still be asked to-day, why should it be the first reaction of the enzyme chain that is affected by narcotics when they inhibit respiration? The answer is that possibly all the stages of the enzyme chain of reactions are surface reactions, but that the

† O. Warburg, *Bioch. Zeitschr.* **189**, 354 (1927) [p. 372].

‡ Kurt H. Meyer and H. Hopff, *Z. f. physiologische Chem.* **126**, 281 (1923); Kurt H. Meyer and H. Hemmi, *Bioch. Zeitschr.* **277**, 39 (1935).

iron system has a special position of its own because it is insoluble in water, whilst the other enzymes in the chain are all soluble. Enzymes dissolved in water are not inhibited by narcotics except when the concentration of the latter is increased to that causing coagulation.

It must, however, be emphasized with regret that the problem of narcotic inhibition has not been as sufficiently studied quantitatively as befits its importance. The chemical problem of respiration has pushed the physical problem, the inhibiting action of narcotics, into the background. In a more complete theory of cell respiration which would deal with the energy changes taking place, the study of narcotic inhibition would take an outstanding place.

CHAPTER VII

DISCUSSION ON OXYGEN TRANSPORTING IRON

In 1922, eight years after the original work was done on oxygen transporting iron, Willstätter, Wieland, and v. Euler met in Leipzig at a symposium on enzymes.† Of the papers read only one dealt with iron 'Für die Konstitution der Peroxydase ist das Eisen belanglos'.‡ This statement, apparently giving information about the constitution of peroxidase, was an attack on the theory of oxygen transporting iron. It was the beginning of a polemic lasting a year, in which Willstätter, Wieland, and v. Euler tried in the following roundabout way to disprove the theory of oxygen transporting iron.

The prospects of isolating the oxygen transporting respiratory enzyme were small since it was not possible to arrange for respiration to take place in solutions. However, there were soluble enzymes which, like those involved in cell respiration, could be specifically and reversibly inhibited by cyanide: peroxidase, catalase and phenol oxidase. The properties of these enzymes appeared favourable for isolation. This isolation was carried out, and it was then shown that these enzymes contained no iron.

In this way the presence of iron in the oxygen transporting enzyme was refuted, since uniformity demanded that all cyanide inhibitions should have a common explanation. [Peroxidase and catalase had been previously embraced by me in the iron theory.§]

Had it been properly carried out, the above plan would have benefited enzyme chemistry. It would have shown that peroxidase and catalase are haem derivatives‖ and, therefore, actually compounds of iron.

But the purest preparations which Willstätter, Wieland, and

† 'Vorträge zur Hundertjahrfeier der Ges. Deutsch. Naturforscher', *Chem. Ber.* **55**, 3583 (1922).

‡ R. Willstätter, *Chem. Ber.* **55**, 3601 (1922).

§ O. Warburg, *Bioch. Zeitschr.* **136**, 266 (1923).

‖ Hugo Theorell, *Ergebnisse der Enzymforschung*, **9**, 231 (1943).

v. Euler's co-worker Hennichs obtained as a result of their isolation attempts did not even show the spectrum of a haem. Therefore their preparations contained negligible amounts of the enzymes. Willstätter and v. Euler believed their preparations were almost pure and even had them analysed. They found that iron was present, but there was no relation between the iron content and the enzyme activity, so that they regarded it as proved that iron was not present as a constituent of either peroxidase or catalase.

1. Willstätter's experiments with peroxidase†

Between 1918 and 1923 Willstätter attempted to isolate peroxidase from horse radish, using adsorption methods.

His most active preparation was 10,000 times as active as the dried root, a value which did not indicate, but which gave the impression of, purity. This impression was still further strengthened in that Willstätter in lectures and in publications did not differentiate as well as one might have wished between 'gereingt' and 'rein'.

In one step in the course of the purification, Willstätter found that the enzyme content increased while the iron content decreased.

	Activity	*Iron content*
Before purification . .	670	0·46%
After purification . .	3,100	0·06%

This is the well-known experiment of Willstätter's from which he drew the conclusion 'iron compounds are closely associated with peroxidase, but the enzyme, however, does not contain the iron as an integral part of its structure'. Willstätter might equally well have concluded from the carbon or nitrogen contents of his preparations, which likewise did not parallel the activity, that the enzyme contained neither carbon nor nitrogen. Strangely enough the iron-free peroxidase was regarded

† R. Willstätter *et al.*, *Liebigs Ann.* **416**, 21 (1908); **422**, 47 (1921); **430**, 269 (1923); *Chem. Ber.* **55**, 3601 (1922).

by science as an important discovery,† although no one could really have had an interest in such a negative finding.

In regard to the activation of hydrogen peroxide by peroxidases the following may be said:

Since iron salts increase catalytically the oxidizing action of hydrogen peroxide to a marked degree, one might have invoked a similar action of the metal in the case of the enzyme. Willstätter's result however, decisively contradicted such a view, since his enzyme preparations, already low in the iron content, became almost iron-free when in their most active state.

The important results of Willstätter's enzyme studies are in disagreement with the hypothesis that the cell peroxidases owe their action to an iron complex.‡

2. Hennichs's experiments on catalase§

The contribution which the Euler Institute made to the discussion on oxygen transporting iron was one from S. Hennichs on 'Aktivität und Eisengehalt hochaktiver Katalasepräparate', which appeared in 1926. Hennichs's starting material was liver, and his highly active preparations contained 4 per cent. iron and 13 per cent. ash. In a comparison of the activity and iron content, these preparations gave the following results:

Activity	*Iron content*
10113	3·67%
11442	3·33%
10872	3·89%
25000	4·12%

The activity and the iron content did not therefore run parallel, thus disproving the iron theory. Like Willstätter, Hennichs was therefore led astray.‖ The high iron content indicated that Hennich's catalase consisted of blood haemin to a large extent. This being so, the fact that the activity and the

† H. Wieland, *Oppenheimers Handbuch der Biochemie*, 2nd ed. **2**, 252 (1923).
‡ H. Wieland and W. Franke, *Liebigs Ann.* **457**, 1 (1927).
§ S. Hennichs, *Chem. Ber.* **59**, 218 (1926).
‖ O. Warburg, ibid. **59**, 739 (1926).

iron content did not run parallel to one another can be readily understood. Actually, catalase contains 0·1 per cent. of iron.† Hennichs found four times as much, yet he tried to prove thereby that catalase contains none.

3. Wieland's experiments on catalase‡

In 1923 Wieland attempted to isolate catalase from liver. His purest solutions were 'completely colourless and clear'. Since, however, very dilute solutions of catalase are red, Wieland's solutions could not have contained very much. He did not give any information as to the iron contents of his purest preparations. His main argument against iron had nothing to do with the isolation experiments, but rather was based on the reversibility of the cyanide inhibition. Thus Wieland found in 1925 what Schönbein§ had already discovered in 1868, namely, that the cyanide inhibition of catalase is reversible. And, just as Schönbein had done in 1868, Wieland concluded that the cyanide inhibition could not be due to chemical reaction between the cyanide and the enzyme. All such reactions of cyanide are reversible.

We believe that the experiments described leave no doubt that the cyanide inhibition of catalase is the result of adsorption, differing only in degree from the effect produced by indifferent gases.

In this case the adsorption theory was invoked to prove that catalase contains no iron.

The results described above disprove, in the case of catalase at any rate, the opinion expressed elsewhere [O. Warburg]‖ that the inhibition of the enzyme action by cyanide is due to a more or less labile complex combination between it and the iron on the surface of the enzyme.

In the same way it might have been proved that haemoglobin contains no iron, since the carbon monoxide compound is also reversible.

† H. Theorell, *Ergebnisse der Enzymforschung*, **9**, 231 (1943).
‡ H. Wieland, *Liebigs Ann.* **445**, 181 (1925).
§ Chr. F. Schönbein, *Journ. prakt. Chem.* **89**, 22, 323 (1863); **105**, 198 (1868).
‖ O. Warburg, 'Über die Adsorptionskonstante der Blausäure',' *Biochem. Zeitschrift*, **119**, 134 (1921).

As further evidence against the presence of iron in catalase Wieland put forward his observation that the methyl ester of prussic acid inhibits catalase. Actually this is not so, but probably Wieland's ester had contained free prussic acid. Even if the experiment had supported Wieland's argument, however, why should the esters of hydrogen cyanide not react with iron ?† Complexes formed by prussic acid esters and iron salts have been known for a long time, and K. A. Hofmann‡ showed that there are cases in which the hydrogen cyanide can be displaced from iron complexes by its esters.

Haemoglobin iron§ also reacts with esters of prussic acid giving dissociating compounds. These will be dealt with in another part of the book.

Amongst the iron catalysts‖ there are some which are, and some which are not inhibited by prussic acid esters. The catalytic action of the iron in blood charcoal and that of the iron combined with cysteine, for example, are inhibited by the ethyl ester of prussic acid, whilst cell respiration and liver catalase are not inhibited.

4. Wieland's experiments on polyphenoloxidase

Somewhat later, in 1928, the work on the third cyanide sensitive enzyme, polyphenoloxidase, appeared. Wieland had attempted without success to isolate it from the potato. Nevertheless he came to the conclusion that this enzyme contained iron, and moreover, that it was a haem compound.††

We think it possible that a part of the catalytic action of the potato oxidase is to be attributed to iron.

This result makes it probable that polyphenoloxidase is also a haem-containing enzyme. Since we have established its sensitivity towards cyanide and carbon monoxide, we may assume that the active iron is present in the ferrous state.‡‡

† O. Warburg, *Chem. Ber.* **59**, 739 (1926).
‡ K. A. Hofmann, ibid. **40**, 1772 (1907).
§ O. Warburg *et al.*, *Bioch. Zeitschr.* **214**, 26 (1929).
‖ S. Toda, ibid. **172**, 17 (1926).
†† H. Wieland and H. Sutter, *Chem. Ber.* **61**, 1060 (1923); **63**, 66 (1930).
‡‡ H. Sutter, *Ergebnisse der Enzymforschung*, **5**, 273 (1936).

In 1937 phenoloxidase was isolated in Dahlem.† It is neither a haem compound, nor does it owe its action wholly or partly to iron salts. Phenoloxidase is a copper protein, related to the haemocyanins, the copper in which transports oxygen by virtue of its valency change cuprous $\leftrightarrows$ cupric.

Wieland and Sutter had failed to observe in their work on the reacting group of phenoloxidase that the carbon monoxide inhibition was insensitive to light. Phenoloxidase must be a heavy metal compound in view of the cyanide and carbon monoxide inhibition, but it cannot be an iron compound because light has no effect on the inhibition by carbon monoxide.

5. Wieland's experiments on iron catalysis

In 1914 I observed that tartaric acid is oxidized at room temperature by molecular oxygen when a small amount of ferrous sulphate is added to the solution‡ and also that the oxidation of dihydroxymaleic acid is accelerated by iron salts.

In 1928 Wieland and Franke§ repeated and confirmed this, at the same time referring to the biological importance of the reaction.

It is too early to assess the biological connexion. Moreover our information with regard to the purely chemical aspect of the action of iron is still too meagre.

Why, however, was the spectrum of the oxygen transporting enzyme which was available in 1928 not sufficient to show the biological relationship? If our purely chemical information of the valency change of iron was considered too meagre, where was there to be found a chemical reaction about which we had less meagre information?

6. Hopkins's remarks‖

Sir Frederick Gowland Hopkins at the twelfth International Physiological Congress summarized the contributions which had been made during the years 1914 to 1926.

† F. Kubowitz, *Bioch. Zeitschr.* **292**, 221 (1937); **299**, 32 (1938).
‡ O. Warburg, *Z. f. physiologische Chem.* **92**, 231 (1914).
§ H. Wieland and W. Franke, *Liebigs Ann.* **464**, 101 (1928).
‖ F. G. Hopkins, *Skand. Arch. f. Physiologie*, **49**, 33 (1926).

If it is true that iron is the oxygen transporting part of the respiratory enzyme, then the reaction between oxygen and iron is subordinate in importance to the activation of the substrates.

At that time information was available only about the iron, and nothing was known of the alloxazine and pyridine proteins, so it seemed right to the scientist Hopkins, who was a romanticist, to rate the unknown as more important than the known. To-day, however, when the whole chain of enzyme reactions in respiration is known, the last link in the chain cannot be regarded as being of more importance than the first.

7. Experiments of H. A. Krebs on papain

L. B. Mendel and A. F. Blood† found in 1910 that the proteolytic enzyme papain is activated by cyanide. The cyanide did not inhibit, but accelerated the action. In 1924 at a time when the discussion on the mode of action of cyanide was in progress, Willstätter‡ put forward the theory that the cyanide activated the papain by forming with it a cyanhydrin.

If this were so, then possibly the solution of the cyanide problem was to be looked for in another direction than that suggested by me. For if hydrogen cyanide formed a cyanhydrin with papain, it was difficult to understand why it did not form cyanhydrins with other enzymes. If the cyanhydrin accelerated the action of this enzyme, why should such a compound not inhibit enzyme reaction in other cases? Heavy metals were apparently no longer necessary to explain the biological action of hydrogen cyanide. Willstätter's theory had two defects. The first was that it had no experimental basis, and the more serious one that it threw no light on the phenomenon which it was supposed to explain. The theory made clear neither why the papain without cyanide is inactive, nor why with cyanide it is active.

Assuming the heavy metal theory of cyanide action to be correct, this was an exception to it, in that the cyanide activated

† L. B. Mendel and A. F. Blood, *Journ. Biol. Chem.* **8**, 177 (1910).

‡ R. Willstätter and W. Grassmann, *Z. f. physiologische Chem.* **138**, 184 (1924).

papain by combining with a heavy metal inhibitor.' This application of our views to papain activation was discussed in 1926 by Myrbäck† and later by Grassmann.‡

In 1930 H. A. Krebs§ studied the problem experimentally at Dahlem. Krebs found that papain is inhibited by traces of heavy metal salts, and that this inhibition is removed by cyanide. He further found that commercial papain can be activated by pyrophosphate, a substance which has only one property in common with hydrogen cyanide, namely, that it forms complexes with heavy metal salts. This important result with pyrophosphate is illustrated graphically. According to my view, this experiment was just as decisive as had been the corresponding experiment with pyrophosphate in the problem of the autoxidation of cysteine.‖

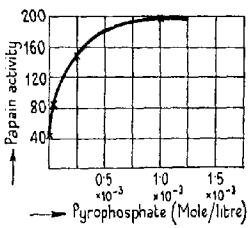


FIG. 5. Activation of papain by pyrophosphate

Since in the hydrolysis by papain only a little enzyme but much substrate is used, the inhibiting heavy metal salts are really introduced with the substrate into the solution. When Krebs freed the gelatin substrate from heavy metal impurities, the gelatin was split by papain, whilst impure gelatin was unaffected by the same enzyme preparation. Krebs therefore by freeing the solution from heavy metal salts could 'activate' papain without the addition of an activator.

Grassmann†† opposed this view of Krebs, and suggested that in the action of cyanide on papain the HS-reaction was involved, though Krebs‡‡ did not consider that an objection. Other objections have been put forward.§§ On the whole, the

† K. Myrbäck, *Z. f. physiologische Chem.* **158**, 231 (1926).
‡ W. Grassmann *et al.*, ibid. **186**, 183 (1930).
§ H. A. Krebs, *Bioch. Zeitschr.* **220**, 281 (1930).
‖ Chapter V, section 5.
†† W. Grassmann, *Z. f. angewandte Chemie*, **44**, 105 (1931).
‡‡ H. A. Krebs, *Bioch. Zeitschr.* **238**, 174 (1931).
§§ E. Maschmann and E. Helmert, *Z. f. physiologische Chem.* **219**, 99 (1933); **220**, 199 (1933).

view of Krebs does not appear to have been accepted,† although no one has put forward a better explanation. Like all objections to the heavy metal theory, these have been predominantly of a negative character.

Krebs is probably right. However, it is possible that there are different degrees of heavy metal poisoning of papain. At first the inhibition may be caused by the combination of the metal and the enzyme, and in the second stage by the oxidizing action of the metal so combined. In the first stage suitable complex-forming substances would reactivate the enzyme, but in the second stage this would only be possible by such substances which were at the same time reducing agents.

† M. Bergmann, J. S. Fruton and H. Fränkel-Conrat, *Journ. Biol. Chem.* **119**, 35 (1937).

CHAPTER VIII

CYTOCHROME

1. The experiments of MacMunn†‡

MacMunn in 1885 discovered by spectroscopic examination that all animal cells 'from echinoderms to man throughout the animal kingdom' contain haem compounds. These are present in two forms according to whether the cell is saturated with, or is cut off from, oxygen. 'They are joined proteids.' 'They are capable of oxidation and reduction and are therefore respiratory.'

Whilst the oxidized haem compounds give no characteristic spectrum with visible light, in the reduced condition they show a four-banded spectrum. MacMunn found that the positions of the bands were about the same in all cells, the mean values being:

605 mμ	*567 mμ*	*550 mμ*	*522 mμ*
yellow-red strong	green strong	green very strong	blue-green weak

In accordance with their presence in muscle or in other tissues, MacMunn called the haem compounds 'myohaematins' or 'histohaematins'. He found the greatest concentration in the wing muscles of rapidly flying insects. MacMunn was able to extract a part of the haems from the cells, and he was surprised that after the extraction they lost their ability to be oxidized by atmospheric oxygen. He observed:

> In studying the chromatology of many invertebrates, I have been struck by the fact that while some of their colouring matters can be reduced by such agents as sulphide of ammonium, yet by shaking with air or by passing a stream of oxygen into them, they cannot be reoxidised; in this point they afford a parallel to the histohaematins. Krukenberg has noticed the same thing, and he has justly concluded that the respiratory process of many of these animals is not as simple a matter as it is supposed to be.

† C. A. MacMunn, *Phil. Trans. Royal Soc. London*, **177**, 267 (1885).
‡ Id., *Journ. Physiology*, **8**, 51 (1887).

Thus there arose at a remarkably early date a problem which has only been solved in recent years, namely, that there are cell pigments which appear to react inside the cell with molecular oxygen and yet are not autoxidizable. To this class many yellow enzymes also belong. The solution to this problem was the enzyme chain. The first link in the chain is always an autoxidizable enzyme, though it may not be recognizable spectroscopically, whilst the oxidation of the later links in the enzyme chain is brought about by those preceding them.

2. Hoppe-Seyler's objections

The views of MacMunn were challenged in 1889 by Hoppe-Seyler's co-worker Levy† who took the view that the myohaematins and histohaematins were substances of no physiological function, but decomposition products of haemoglobin. MacMunn‡ replied quite rightly that the histohaematins were also to be found in animals having no haemoglobin and that their concentration was greatest in the muscles of insects without haemoglobin. But Hoppe-Seyler§ thought MacMunn's reply invalid. The conditions in the lower forms of animal life might not apply to the higher animals.

I have reported this discussion because it shows how dangerous it is when people allow themselves to be influenced by false objections. MacMunn remained silent, and the result was that nothing more was heard of histohaematins during the next 33 years.

3. Experiments of Hans Fischer

MacMunn's discovery that not only the red blood cells, but that all cells contained haems was verified analytically in the years 1923 and 1924 by Hans Fischer.‖ 'I am convinced of the correctness of MacMunn's results.' Fischer's starting point was porphyria, a disease of man in which large amounts of porphyrin

† L. Levy, *Z. f. physiologische Chem.* **13**, 309 (1889).
‡ C. A. MacMunn, ibid. **13**, 497 (1889); **14**, 328 (1890).
§ F. Hoppe-Seyler, ibid. **14**, 106 (1890).
‖ Hans Fischer, *Strahlentherapie*, **18**, 185 (1924).

are excreted in the urine and faeces. In 1916 Fischer† isolated a porphyrin which he called coproporphyrin I. He proved that the substance was different from the porphyrin of the blood haem, protoporphyrin. Coproporphyrin I is tetramethyl-porphin-tetrapropionic acid; protoporphyrin is dimethyl-divinyl-porphin-dipropionic acid. The arrangement of the methyl or vinyl groups and the propionic side chains in the two porphyrins indicated that the one could not have been formed from the other. Hans Fischer therefore concluded that there are either red blood pigments the haems of which are different, or there must occur naturally haem compounds which are not blood pigments. The connexion with the work of MacMunn was thus established.

The question as to how coproporphyrin I arises remains still unanswered to-day, but the discovery of two naturally occurring porphyrins started Fischer‡ looking for haems everywhere in living nature. He thus discovered that yeast contains haems, and moreover, to such an extent that if a pyridine extract is made of a little fresh baker's yeast, it appears quite red. Fischer also found haems in plant cells and he isolated the corresponding porphyrins.

4. Cytochrome§

Keilin's work in 1925 confirmed the results of the spectroscopic studies of MacMunn, and he attempted, moreover, to show‖ the connexion between MacMunn's work and the oxygen transporting iron of 1924, embodying ideas of Meyerhof and Harrison on the haem nature of the enzyme.

Although Keilin in his work of 1925 erroneously identified MacMunn's histohaematin with the oxygen transporting enzyme, this work was nevertheless of great importance in the solution of the respiration problem. Keilin then gave MacMunn

† Hans Fischer, *Münchener Med. Wochenschr.* 1916, p. 377 and 1923, p. 1143.

‡ Hans Fischer *et al.*, *Z. f. physiologische Chem.* **135,** 253; **138,** 288; **140,** 57 (1924); **144,** 101 (1925).

§ D. Keilin, *Proc. Royal Soc. London,* B **98,** 312 (1925); **100,** 129 (1926); **104,** 206 (1929); **106,** 418 (1930).

‖ Cf. chap. VI, section 4.

his rightful place. He showed, however, that haems are more widely distributed in nature than MacMunn had believed. He confirmed the existence of Fischer's yeast haem using the spectroscopic method, and by applying the 'inhibition technique' to histohaematin, he paved the way to the recognition of these haem compounds as being links in the chain of respiration enzymes. It was, however, unnecessary to change the name of MacMunn's histohaematin into cytochrome. Keilin replaced a name which correctly expressed the chemical constitution by a name which did not differentiate the histohaematin from other cell pigments. We shall, however, use the name cytochrome in accordance with Keilin's wishes.

Keilin attributed three of the MacMunn bands to three different haem compounds which he called cytochrome *a*, cytochrome *b*, and cytochrome *c*.

The fourth MacMunn band (522 mμ) ought to be common to all three cytochromes. Actually the band at 522 on closer examination appears to consist of two or three separate bands as MacMunn had previously observed. Nevertheless it is probable that the 522 band belongs to cytochrome *c* and that the corresponding bands for cytochrome *a* and *b* cannot be seen on spectroscopic examination of cells owing to their low concentration.

All four MacMunn bands are so-called secondary bands. The main bands which are situated in the blue region were discovered by us in 1931 using blue light.† We found in the blue region bands at 417 mμ, 433 mμ, and 449 mμ. Of these, however, the last, as Keilin and Hartree‡ showed in 1939, belongs probably in part to the oxygen transporting enzyme.

To summarize: Cytochrome consists of three haem compounds which in the reduced or ferrous condition show the following bands:

	Cytochrome a	*Cytochrome b*	*Cytochrome c*
1. Secondary bands	605 mμ	567 mμ	550 mμ
2. Secondary bands	?	?	522 mμ
3. Main bands	449 mμ	433 mμ	417 mμ

† O. Warburg and E. Negelein, *Bioch. Zeitschr.* **233**, 486 (1931); **238**, 135 (1931).
‡ D. Keilin and E. F. Hartree, *Proc. Royal Soc.* B **127**, 167 (1939).

5. The experiments of Anson and Mirsky†

Anson and Mirsky in 1925 showed that the haemochromogens are dissociating compounds of ferrohaem (ferroporphyrin) with nitrogen-containing bases, such as ammonia, pyridine, and protein:

$$\text{ferrohaem} + \text{base} \rightleftharpoons \text{haemochromogen}.$$

Whilst the ferrohaems have no characteristic spectra, the haemochromogens are distinguished by sharp band spectra, the positions of the bands being determined by both components, the haem and the base. The same haem forms different haemochromogens according as it is coupled with ammonia, with pyridine, or with a protein. Different proteins coupled with the same haem give different haemochromogens distinguishable by the position of the bands.

Since the haemochromogens are dissociating compounds, any haemochromogen can be converted into another by reaction with a nitrogenous base. If the affinity and the concentration of the added base are adequate a new haemochromogen is formed by displacement.

On account of its great affinity for haem, pyridine is a particularly active substance in this respect. If a haemochromogen is dissolved in concentrated aqueous pyridine, the whole of the haem is converted into pyridine haemochromogen. Anson and Mirsky applied this to the cytochrome problem. They came to the conclusion that the haems of cytochrome *b* and *c* were identical and, moreover, that it was protohaem. The bands of cytochrome *b* and *c* are different, therefore, not on account of the haem, but on account of the protein component. Further, cytochrome *a* differs from cytochrome *c* in respect of both the haem and the protein. All that has been discovered since then is in agreement with this conclusion.

6. Oxidation and reduction of cytochrome

A stoppered glass vessel with plane parallel sides and of about 200 c.c. capacity and 2 cm. internal width is two-thirds filled with a 20 per cent. suspension of baker's yeast which has been

† M. L. Anson and A. E. Mirsky, *Journ. Physiology*, **60**, 50, 161, 221 (1925).

previously washed with sodium chloride solution. On the near side of the vessel the filament of a powerful metal-filament lamp is arranged, and on the other side a spectroscope is fitted up. If nitrogen is passed through this yeast suspension or if the suspension is allowed to stand undisturbed, the bands of reduced cytochrome can be seen. If, however, oxygen is passed through the suspension, the bands disappear because saturation of the cells with oxygen converts the cytochrome into the oxidized form. In this way it can be seen how cytochrome in respiring cells is oxidized and reduced. As MacMunn said, 'They are capable of oxidation and reduction and are, therefore, respiratory.'

7. Inhibition of cytochrome action by cyanide

Keilin studied the oxidation and reduction of cytochrome using the same methods which we had employed for cell respiration. He found that the two processes were completely analogous. We found that cyanide and hydrogen sulphide inhibit yeast respiration: Keilin found that the action of yeast cytochrome was likewise inhibited by the same concentrations. We found that hydrogen cyanide ethyl ester and pyrophosphate, in spite of their being complex-forming substances, do not inhibit yeast respiration; Keilin found that these compounds did not inhibit the action of yeast cytochrome. We found that indifferent narcotics inhibit yeast respiration by a different mechanism from that of cyanide; Keilin found that this was also the case for the action of yeast cytochrome.

In view of this, anyone would have drawn the conclusion that cytochrome is the oxygen transporting enzyme. However, certain points of disagreement arose when the inhibition of the cytochrome action was more closely investigated, for example with cyanide.

If oxygen is passed through a yeast suspension till the absorption bands of cytochrome disappear and cyanide in sufficient quantity (N/10,000) to inhibit the respiration is added, the bands of the reduced cytochrome reappear in spite of the oxygen, and, moreover, these bands are exactly the same as

those to be seen in the absence of cyanide and oxygen. Cyanide, therefore, appeared to inhibit the oxidation of cytochrome without reacting with it—a contradiction in terms.

8. Keilin's theory†

Keilin did not consider this anomaly, but on the grounds of the concordant behaviour of cell respiration and cytochrome function towards inhibiting agents, he put forward the theory that cytochrome was the oxygen transporting enzyme. He replaced the iron in our scheme of 1924 by cytochrome iron.

$O_2 \rightarrow$ ferro-cytochrome $\rightarrow$ ferri-cytochrome $\rightarrow$ substrate.

'Cytochrome is oxidized by the air and reduced by the tissue itself.' 'The oxygen is constantly taken up by this pigment and given up to the cells.'

The cytochrome, according to this theory, was therefore autoxidizable and, moreover, sufficiently so to transfer oxygen in respiration.

9. Inhibition by carbon monoxide‡

In 1926 we examined the action of carbon monoxide on yeast cytochrome and found that it inhibited the oxidation of cytochrome iron at concentrations which were the same as those inhibiting respiration.

In this case, too, as with the cyanide in Keilin's experiments, the carbon monoxide did not affect the cytochrome bands. This substance, therefore, also appeared to inhibit the oxidation of cytochrome without reacting with it.

10. The Warburg theory

If it were assumed that in respiring cells the oxygen transporting enzyme oxidized the cytochrome, all difficulties would be eliminated.

$O_2 \rightarrow$ oxygen transporting enzyme $\rightarrow$ cytochrome $\rightarrow$ substrate.

Cytochrome, although not autoxidizable, would be immediately oxidized when oxygen was present in the respiring cell, but only

† D. Keilin, *Proc. Royal Soc.* B **98**, 312 (1925).

‡ O. Warburg, *Bioch. Z.* **177**, 471 (1926), and A. Reid, ibid. **242**, 159 (1931).

through the agency of the oxygen transporting enzyme. Moreover, cyanide and carbon monoxide, although they did not react with the cytochrome, would inhibit its oxidation by their reaction with the oxygen transporting enzyme.

This theory was put forward by me at the Royal Society† in London on 12 May 1927, and by Keilin‡ in Paris on 27 May 1927. It was generally accepted. The discussion which then arose with Keilin and which is dealt with in Chapter XVI concerned the further question: What is the oxygen transporting enzyme? Is it an iron compound or a haem compound or neither?

11. The sequence of the components§

If the three cytochrome components in the respiring cell are arranged in an oxidation-reduction chain, the question arises as to the sequence of the components. Eric G. Ball in 1938 attempted to answer the question by the following experiment:

A suspension of washed heart muscle was treated with enough hydrosulphite to allow the bands of the three ferrocytochromes to appear in full strength. By preventing access of oxygen, the oxygen transporting iron and also the reoxidation of the ferrocytochrome were excluded. Then, oxidation-reduction systems of different potentials were added and their effect on the ferrocytochrome bands observed. Large differences in the effect on the three cytochromes were seen. For example, the methylene blue system caused disappearance of only cytochrome *b* bands. With systems of considerably higher oxidation potentials only were the bands of cytochrome *a* and *c* made to disappear.

In this way Ball determined the oxidation potential E_0 (potential at which [ox.] = [red.]) of the three cytochromes and found:

	Cytochrome a	*Cytochrome b*	*Cytochrome c*
$E_0 =$	+0·29 volt	−0·04 volt	+0·27 volt

Ball concluded, therefore, that in the respiring cell, cytochrome

† O. Warburg, *Die Naturwissenschaften*, **15**, 546 (1927).
‡ D. Keilin, *Soc. de Biologie Paris, Réunion Plénière*, 27 and 28 May 1927.
§ Eric G. Ball, *Bioch. Zeitschr.* **295**, 262 (1938).

a oxidized cytochrome *c*, which in its turn oxidized cytochrome *b*, so that the sequence of the iron atoms in the chain would be

$$O_2 \rightarrow \text{Oxygen transporting Fe} \rightarrow Fe_{\text{cytochrome } a} \rightarrow Fe_{\text{cytochrome } c} \rightarrow Fe_{\text{cytochrome } b}.$$

If in the stationary state of respiration cytochromes *a* and *c* were present almost completely in the reduced state, whilst cytochrome *b* was almost completely in the oxidized state, then the E_0 values would not determine the sequence of the cytochromes since

$$E = E_0 + RT \ln \frac{[\text{ox.}]}{[\text{red.}]}.$$

The sequence could therefore be different from that postulated by Ball. But under such conditions in the resting state of the respiration the ferro bands of cytochrome *a* and *c* would have to be fully developed, whilst that of cytochrome *b* need be hardly visible. Actually the situation appears to be just the reverse. The theory of Ball is, therefore, probably correct.

12. Rate of cytochrome reduction†

If a yeast suspension takes up A moles of oxygen per minute, and v mole of cytochrome component are reduced per minute, then if the whole respiration proceeds over the cytochrome,

$$A = \tfrac{1}{4}v,$$

since $\frac{1}{4}$ mole oxygen is necessary to reoxidize 1 mole cytochrome.

Using 1 c.c. bakers' yeast at 0° we found

$$A \equiv 0{\cdot}34 \text{ c.mm. oxygen per minute,}$$

$$v \equiv 4 \times 0{\cdot}32 \text{ c.mm. cytochrome per minute.}‡$$

Thus the expected relationship between the oxygen requirement and the cytochrome reduction was realized.

It was shown by this experiment that the route over cytochrome is not just one of alternative routes, but that it is the main route by which the oxidation equivalent of the respired oxygen is carried forward.

Note. The rather cumbersome arrangement of the experiment which we described in 1934 can be simplified in the following way:

† E. Haas, *Die Naturwissenschaften*, **22**, 207 (1934).
‡ 1 millimole Fe = 22,400 c.mm.

The spectra of two similar yeast suspensions through which oxygen is continuously passed are projected one over the other. To suspension I, which serves as a control, a small measured amount of ferrocytochrome *c* is added. This, in spite of the oxygen, remains reduced as it does not penetrate into the cells and is not autoxidizable in solution. To suspension II there is added at a time t_0 enough hydrogen cyanide to inhibit completely the respiration. The band at 550 mμ then appears in II also, and the intensity increases until at a time t it has reached that in suspension I. If n mole ferrocytochrome had been added to I, then the cells in suspension II must have reduced n mole ferricytochrome to ferrocytochrome in a time $t-t_0$.

The experiment must be arranged so that the amount of cytochrome added to I is not greater than one-third of the amount present in the yeast cells. Only then does the initial rate of cytochrome reduction correspond to that of the respiration in the resting state.

13. Cytochrome *c*†

Of the three cytochromes, the components *a* and *b* are so firmly combined with the insoluble cell constituent that it was not possible in the past to dissolve them out. Contradictory statements are to be ascribed to the fact that the insoluble material, particularly in alkaline media, forms suspensions which are difficult to centrifuge and which appear to be solutions.

Cytochrome *c* which is soluble in water was isolated by Hugo Theorell. It is a conjugated protein of molecular weight 13,000 and contains 1 molecule of protohaem which corresponds to the determined iron content of 0·43 per cent.

In contrast to haemoglobin, cytochrome cannot be reversibly broken down into the haem and protein. This is because the haem and the protein are joined together by two strong bonds, which one can visualize as being formed by the union of two

† Hugo Theorell, *Bioch. Zeitschr.* **279**, 463 (1935); **285**, 207 (1936). Hugo Theorell and Akeson, *Journ. Amer. Chem. Soc.* **63**, 1804 (1941). Hugo Theorell, *Ergebnisse der Enzymforschung*, **9**, 231 (1943).

cysteine residues in the protein to the vinyl groups in the haem, thus giving two thioether linkages. On mild acid hydrolysis the di-cysteine compound of the protoporphyrin is split off, but on strong acid hydrolysis, haematoporphyrin is obtained, as was shown in 1933 by Zeile and Reuter.

The colour changes of cytochrome which Theorell observed in the titration of the acid groups and which he determined colorimetrically are explained by the reversible action between the iron of the cytochrome and the imidazole groups of the protein, since the colour of the cytochrome during the titration changes just at those pH ranges at which the titratable acidity could only correspond to imidazole residues.

CHAPTER IX

ACTION OF CARBON MONOXIDE ON CHEMICAL PROCESSES IN CELLS

In the middle of last century Claude Bernard† discovered that the poisonous action of carbon monoxide is due to its action on haemoglobin. 'On voit par ces résultats que l'oxyde de carbone déplace l'oxygène du sang.' The equation for the displacement is:

$$HbO_2 + CO \rightleftharpoons HbCO + O_2;$$

and therefore the equilibrium may be expressed as

$$\frac{HbO_2}{HbCO}\frac{CO}{O_2} = K.$$

If CO/O_2 gives the relation of the gases in the surrounding atmosphere, then, at 37°, K has a value of the order of 1/200. The haemoglobin is therefore combined to the same extent with oxygen and carbon monoxide when the relative concentrations are given by $CO/O_2 = 1/200$. This condition is reached with air when it contains 0·1 per cent. carbon monoxide. At approximately this concentration of carbon monoxide in inspired air, toxic symptoms appear in animals which have haemoglobin, because half of their haemoglobin is cut off from oxygen transport and the remainder is not sufficient to supply the tissues with oxygen.

In 1895 John Haldane‡ showed that a mouse will remain alive at a carbon monoxide pressure of 1 atmosphere if the oxygen pressure is 2 atmospheres. Under these conditions the haemoglobin of the mouse is completely combined with carbon monoxide, but the oxygen dissolved in the blood is sufficient to keep the tissues supplied.

Haldane's experiment appears to confirm completely the view of Claude Bernard that carbon monoxide is a blood poison but not a cell poison: 'Apart from its action in putting the red cells out of action as oxygen-carriers carbonic oxide would thus appear to be a physiologically indifferent gas like nitrogen.'

† Claude Bernard, *Substances Toxiques*, Paris, 1857.

‡ John Scott Haldane, *Journ. Physiology*, **18**, 201 (1895).

1. Action of carbon monoxide on cell respiration†

In 1926 we found that carbon monoxide inhibits cell respiration. Bakers' yeast suspended in phosphate solution containing glucose is introduced into the main compartment of a conical-shaped manometer vessel. The internal compartment contains potassium hydroxide to absorb the carbon dioxide produced by respiration and fermentation. The air space is filled with carbon monoxide and oxygen, or with nitrogen and oxygen. Effects due to the variation of oxygen pressure are thus eliminated, because the respiration in the two experiments is compared at the same oxygen pressure in the presence of carbon monoxide or nitrogen. 'Inhibition' in the following table represents, therefore, inhibition compared with the nitrogen control. I found, for example,

No.	*Temperature °C.*	*Gas mixture in volume %*		*Inhibition of the respiration*
		CO	O_2	
1	20	80·1	19·9	35
	20	88·6	11·4	61
2	37·5	80·7	19·3	38
	37·5	87·7	12·3	60
3	37·5	78·0	22·0	22
	37·5	90·6	9·4	55
4	20	78·8	21·2	24
	20	79·0	4·4	72
5	37·5	76·8	23·2	34
	37·5	74·7	5·3	77
6	37·5	80·0	20·0	36
	37·5	80·0	4·4	74

In Experiments 4 to 6 the oxygen pressures vary at constant carbon monoxide pressures. As can be seen, the inhibition of respiration at the same carbon monoxide pressure becomes greater with decreasing oxygen pressure. This means that the action of carbon monoxide on respiration does not depend on the carbon monoxide pressure but on the relationship of the carbon monoxide pressure to that of the oxygen. This was an important result, from which it follows that the carbon monoxide attaches itself to the enzyme molecule at the point which under normal conditions is the site of the oxygen reaction. The value

† O. Warburg, *Bioch. Zeitschr.* **177**, 471 (1926).

of the ratio CO/O_2 which produces half inhibition of the respiration is generally about 10, whilst as mentioned above, the ratio CO/O_2 which brings about half displacement of the oxygen from haemoglobin has a value of about 1/200. If the oxygen transporting enzyme reacts qualitatively with carbon monoxide and oxygen like haemoglobin, the properties of the enzyme and haemoglobin are so different from a quantitative point of view that there is no question of their being identical.

Of those who worked on the physiological action of carbon monoxide, John Haldane was nearest to discovering that carbon monoxide is also a cell poison. The mouse which remained alive under a pressure of two atmospheres of oxygen and one of carbon monoxide might have been killed by the reaction of carbon monoxide on the body cells if Haldane had increased the carbon monoxide pressure by a further two atmospheres. J. B. S. Haldane carried out this experiment in 1927: 'It is clear that CO has a poisonous action apart from its combination with haemoglobin.'†

2. Methods

In the investigation of the action of carbon monoxide on respiration the following points must be taken into consideration:

1. Dilute aqueous potash absorbs carbon monoxide with the formation of potassium formate:

$$CO + KOH = HCOOK.$$

For example we found‡ that 1 c.c. 5 per cent. potassium hydroxide at a carbon monoxide pressure of 1 atmosphere absorbs per hour:

at 20° . . .	2 c.mm. CO
at 37° . . .	17 c.mm. CO

If, therefore, the carbon monoxide inhibition of respiration is determined in our simple manometric apparatus with potassium hydroxide in the inner compartment, a control experiment

† J. B. S. Haldane, *Bioch. Journ.* **21**, 1068 (1927).
‡ O. Warburg and co-workers, *Bioch. Zeitschr.* **242**, 170 (1931).

without cells must be set up and the volume change subtracted from the respiration value obtained using the same carbon monoxide-oxygen mixture.

2. Just as cells are able to increase the rate of the reaction

$$CO_2 + H_2O = H_2CO_3$$

it appears that they can also accelerate the reaction

$$CO + H_2O = HCOOH.$$

In experiments with feebly respiring cells such as red blood cells, the effect of this reaction becomes appreciable in relation to the respiration. In such cases a control vessel containing cells is set up, the gas space of which contains the same amount of carbon monoxide as in the experimental vessel, but no oxygen. This control gives the carbon monoxide absorbed by the potassium hydroxide and also by the cells, the necessary correction being made to the respiration values obtained with carbon monoxide.

3. If the respiration is determined at a total gas pressure of 1 atmosphere, as is usual in our manometric technique, then when the ratio CO/O_2 is large, the oxygen pressure in the gas space must be small. Under these conditions the oxygen pressure in the cells, in which the oxygen is used up and into which it must diffuse, will be considerably smaller than in the gas space. The ratio CO/O_2 within the cell, which alone controls the reaction, is therefore usually unknown. The question as to how the respiration will change with change in the ratio CO/O_2 cannot therefore be quantitatively studied with the usual manometric arrangements. The following is a suitable arrangement,† however: the vessel is made to rotate about a vertical axis at about 650 revolutions per minute. The cell suspension then rises up on the walls of the vessel and is rotated as a thin layer. The pressure change due to respiration is read off during the rotation using a cathetometer microscope with an accuracy of 0·05 mm. With this arrangement we could increase the value of the ratio CO/O_2 to 1,000.

The measurement of carbon monoxide inhibition of respiration

† O. Warburg and F. Kubowitz, *Bioch. Zeitschr.* **214**, 19 (1929).

at definite oxygen pressures is therefore not easy. The experimental material must be small unicellular organisms and, moreover, such as are not harmed by the high degree of agitation.

4. We have found† that carbon monoxide can be oxidized in aqueous solution by molecular oxygen to give carbonic acid if small amounts of certain haems are added to the solutions. It appears that catalytic oxidation of carbon monoxide also takes place in cells, and on this account the respiration-inhibiting action of the carbon monoxide can be completely or partly masked. Per Eric Lindahl‡ has suggested that it would be possible to ascertain in inhibition experiments the extent of this carbon monoxide oxidation, which he assumes is the same in light as in darkness, by strongly irradiating the cells in carbon monoxide-oxygen mixtures. Under these conditions the inhibition of the respiration would disappear, but the combustion of the carbon monoxide would go on, so that, from the increase of respiration over the normal uninhibited respiration, the extent of carbon monoxide oxidation could be calculated.

On the whole, the problem of the oxidation of carbon monoxide in respiring cells has not been methodically worked out. It appears certain that cells can bring about the absorption of carbon monoxide; whether there is really a hydration or an oxidation of the carbon monoxide is not yet certain.

3. The partition equation

Since for the partition of haemoglobin between oxygen and carbon monoxide the equation

$$\frac{HbO_2}{HbCO}\frac{CO}{O_2} = K_{Hb}$$

is applicable, on the grounds of analogy we can assume that in the partition of the oxygen transporting enzyme the corresponding equation

$$\frac{FeO_2}{FeCO}\frac{CO}{O_2} = K \qquad (1)$$

will hold.

A verification of this equation by chemical analysis as in the

† E. Negelein, *Bioch. Zeitschr.* **243,** 386 (1931).

‡ Per Eric Lindahl, *Zeitschrift f. vergleichende Physiologie*, **27,** 316 (1939).

case of haemoglobin is not possible. But if the assumption is made that in mixtures of carbon monoxide and oxygen the observed respiration is proportional to the amount of enzyme not combined with carbon monoxide, the ratio $FeO_2/FeCO$ can be calculated from the respiration inhibition.

If A_0 represents the non-inhibited respiration and A the respiration inhibited by carbon monoxide, and

$$FeO_2 + FeCO = \sum Fe,$$

then
$$\frac{A}{A_0} = n = \frac{FeO_2}{\sum Fe}$$

and
$$\frac{A_0 - A}{A_0} = (1-n) = \frac{FeCO}{\sum Fe},$$

then
$$\frac{n}{1-n} = \frac{FeO_2}{FeCO},$$

and instead of (1) we can write

$$\frac{n}{1-n}\frac{CO}{O_2} = K, \qquad (2)$$

in which on the left-hand side we now have only values which can be determined experimentally. To verify the equation it is only necessary to determine the respiration for various values of the ratio CO/O_2 and to see if $\frac{n}{1-n}\frac{CO}{O_2}$ remains constant.

Using this method we found with Torula yeast† at 10°:

$\frac{CO}{O_2}$	*Pressure change in 20 minutes*	$n = \frac{p}{p_0}$	$\frac{n}{1-n}\frac{CO}{O_2} = K$
0	$p_0 = -1{\cdot}85$ mm.	..	..
23	$p = -0{\cdot}90$ mm.	0·49	21
83	$p = -0{\cdot}40$ mm.	0·22	23
440	$p = -0{\cdot}10$ mm. (calculated $-0{\cdot}09$ mm.)	..	..
1000	$p = 0$ (calculated $-0{\cdot}04$ mm.)	..	..

When, therefore, the ratio CO/O_2 was increased from 23 to 83, $\frac{n}{1-n}\frac{CO}{O_2}$ remained constant with a value of $K = 22$. For $CO/O_2 = 440$ and $CO/O_2 = 1000$ the respiration was so small that K could not be calculated. If we substitute for K the value

† O. Warburg and F. Kubowitz, *Bioch. Zeitschr.* **214**, 19 (1929).

22 and use it to calculate the pressure changes p, the calculated p values agree within the error of measurement (0·05 mm.) with the values found.

$\frac{n}{1-n}\frac{CO}{O_2}$ is neither constant for all types of cells, nor for the same cells under all conditions of culture. For example, we found constant K values in the case of Torula yeast from old cultures, but for the same Torula strain from younger cultures—the cells in which respired more strongly—lower and less constant values were obtained

	$\frac{CO}{O_2}$	$n = \frac{A}{A_0}$	$\frac{n}{1-n}\frac{CO}{O_2}$
40-hour culture . .	19	0·065	29
	68	0·306	30
14-hour culture . .	4	0·70	9·3
	19	0·27	7·0

In general, according to our results, there are cells for which the partition equation applies just as well as for haemoglobin. This is remarkable in view of the assumption made in deriving the expression $\frac{n}{1-n}\frac{FeO_2}{FeCO}$, and in view of the fact that the partition equation could only apply if four chemical reactions of the oxygen transporting enzyme in the respiring cell proceeded as uniformly as if the enzyme were present in pure solution.

Cells for which the partition equation is applicable have been of use to us for the determination, from the photochemical action, of the molar absorption coefficient of the oxygen transporting enzyme. For the determination of the relative absorption spectrum of the enzyme, on the other hand, it is immaterial whether the partition equation applies or not.

In many investigations of the action of carbon monoxide on respiration, too much importance has, in my view, been attached to the partition equation. This may be attributed to haemoglobin. On the other hand, there is nothing more important from the point of view of the physiology of haemoglobin and from the point of view of carbon monoxide poisoning in man and the higher animals than the laws governing the combination of oxygen and

carbon monoxide with the blood pigment. In so far as our problem, the chemical mechanism of cell respiration, is concerned, the essential factors are that carbon monoxide inhibits this respiration, that this inhibition depends on the ratio CO/O_2, and that it is reversed by the action of light. Whether and to what extent the expression $\frac{n}{1-n}\frac{CO}{O_2}$ is constant does not matter.

4. The action of light on carbonylhaemoglobin†

In determining the partition of haemoglobin between oxygen and carbon monoxide, Haldane and Smith happened to find that, for the same blood and the same gas mixture, the partition varied according to the time of the year. Blood saturated with a definite gas mixture contained more carbonylhaemoglobin in winter than in summer. As the partition determinations were carried out colorimetrically in daylight, Haldane and Smith thought that perhaps it might be the varying intensity of the light which brought about the variation in the partition. Actually, measurements of the partition in direct sunlight showed that no carbon monoxide had combined, whilst the colour of the carbonylhaemoglobin immediately appeared when the measurements were made in diffused light. 'The experiment could be repeated again and again, the colour alternately appearing and disappearing, so that it was evident that the carbonic oxide was not destroyed, but remained in the air of the bottle. Its affinity for the haemoglobin must have been diminished by the sunlight to at least a twentieth.'

In this way Haldane and Smith discovered the photochemical dissociation of the carbon monoxide-iron compounds.

5. Action of light on other carbon monoxide-iron compounds

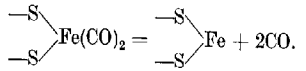
Probably all carbon monoxide-iron compounds are light-sensitive. Iron pentacarbonyl‡ is decomposed by light according to the equation

$$2Fe(CO)_5 = Fe_2(CO)_9 + CO.$$

† John Haldane and J. Lorraine Smith, *Journ. Physiology*, **20**, 497 (1896).

‡ J. Dewar and Jones, *Proc. Royal Soc.* A, **76**, 558 (1905); **79**, 66 (1907).

The carbon monoxide compounds of ferrous sulphydryl compounds† decompose in light according to the equation



On the other hand, up till now it has not been possible to decompose by light a carbon monoxide compound in which the carbon monoxide was joined to any metal other than iron. For example, the following are not light-sensitive: the carbon monoxide compounds of nickel, of nickel cysteine, of cobalt cysteine, of cuprous chloride, and of the copper in the haemocyanins. These results are important on account of their application to enzyme chemistry. Reversible inhibition of an enzyme reaction by carbon monoxide indicates that the reacting group of the enzyme is a heavy metal. If the carbon monoxide inhibition is light-sensitive, then iron is the heavy metal. We have found no exception to this rule.

6. Action of light on the carbon monoxide inhibition of respiration‡

A 75-watt metal filament lamp is placed under two conical manometric vessels which are shaken in a thermostat. Each vessel contains 2 c.c. of a dilute yeast suspension. The gas space contains nitrogen and oxygen or carbon monoxide and oxygen. The light is switched on and off for periods of 20 minutes.

In Fig. 6, results of such an experiment are illustrated graphically. As can be seen, the light in the nitrogen-oxygen mixture has no effect on the respiration. The respiration inhibited by carbon monoxide, however, increases with the light and decreases in the dark. This means that the carbon monoxide compound of the oxygen transporting enzyme is decomposed by light, just as was the case with carbonylhaemoglobin. In order to verify the influence of wave-length on the reaction we selected four regions of the spectrum, made their intensities the same and irradiated yeast cells, the respiration of which had

† W. Cremer, *Bioch. Zeitschr.* **194,** 231 (1928); **206,** 228 (1929); H. Hartmann, ibid. **223,** 489 (1930); F. Kubowitz, ibid. **282,** 277 (1935).

‡ O. Warburg, ibid. **177,** 471 (1926).

been inhibited by carbon monoxide. Thus with light of the same intensity but different wave-length, we found:

In the blue (436 mμ)	strong action
In the green (546 mμ)	weak action
In the yellow (578 mμ)	weak action
In the red (700–750 mμ)	no action

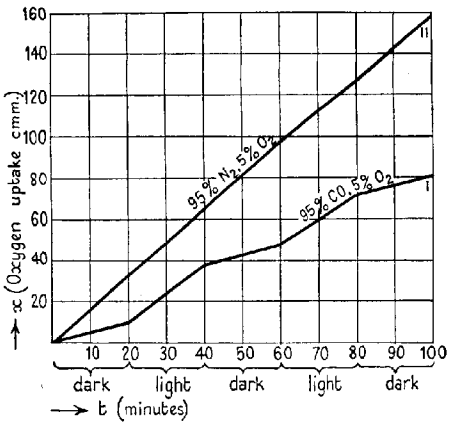


FIG. 6. Action of light on the carbon monoxide inhibition of yeast respiration.

This is the experiment which gave rise to the method for the determination of the absorption spectrum of the oxygen transporting enzyme.

7. Other experimental material

The inhibition of respiration by carbon monoxide, and the action of light on the carbon monoxide inhibition were discovered in experiments with yeast cells. The yeast cell is not, however, unique in this respect. Most aerobic cells which we have investigated have behaved in a similar way to yeast.

Amongst cells of the higher animals which, for technical reasons, are suitable material for study we have the following: blood platelets, the white cells of bone marrow,† and the strongly respiring

† A. Fujita, *Bioch. Zeitschr.* **197**, 189 (1928).

red blood cells found in phenylhydrazine anaemia.† Amongst cells of lower animals we may mention sea urchins' eggs whose behaviour towards carbon monoxide and light has been thoroughly investigated by John Runnström.‡ A plant cell which, according to the work of Robert Emerson,§ behaves towards carbon monoxide and light like the yeast cell is the heterotrophic Chlorella.

The investigation of tissues is more difficult than that of unicellular organisms on account of the difficulty of supplying oxygen to the cells at the low oxygen tensions at which carbon monoxide inhibition can be realized. Nevertheless, we have been able in qualitative experiments by the irradiation of thin animal tissue or of thin sections of tissues‖ to show that they behave like yeast cells towards carbon monoxide and light. We have tried the following tissues: liver, chorion, retina, embryos of the chicken and the rat, and rat sarcoma. Using the retina it was possible to determine some points on the absorption spectrum of the oxygen transporting enzyme from the action of light on the carbon monoxide inhibition.††

Francis O. Schmitt‡‡ has shown that, in the case of nerve, carbon monoxide inhibits the respiration, that the inhibition becomes smaller on exposure to light, and that in mixtures of carbon monoxide and oxygen the action current of nerve increases on exposure.

8. Red blood cells

The normal red blood cells of rabbits have a very small aerobic respiration. If the cells are saturated with air and kept at 38° in the absence of oxygen, it takes 40 hours till the cells have used up the oxygen in their oxyhaemoglobin. If rabbits are injected over a long period with phenylhydrazine, the normal red blood cells are replaced by those having a respiration about 40 times greater, as Morawitz§§ found.

† O. Warburg *et al.*, ibid. **242**, 170 (1931).
‡ J. Runnström, *Protoplasma*, **10**, 106 (1930).
§ R. Emerson, *Journ. General Physiology*, **9**, 469 (1927).
‖ O. Warburg, *Bioch. Zeitschr.* **189**, 354 (1927).
†† O. Warburg and E. Negelein, ibid. **214**, 101 (1929).
‡‡ Francis O. Schmitt, *Amer. Journ. Physiology*, **95**, 650 (1930).
§§ P. Morawitz, *Arch. Exper. Path. and Pharm.* **60**, 298 (1909).

If the action of carbon monoxide on red blood cells is investigated manometrically without special control experiments, it is found† that the strongly respiring cells like yeast cells are inhibited by carbon monoxide. $\frac{n}{1-n}\frac{CO}{O_2}$ has a value of about 10.

The respiration of the normal (weakly respiring) red blood cells appears not to be inhibited, however, under the same conditions—in fact it appears to be increased. The pressure reductions in carbon monoxide-oxygen are then greater than in nitrogen-oxygen.

If the respiration, however, of the normal red blood cells is determined using all the controls recommended in section 2 of this chapter, then it is observed that the respiration of the normal cells is inhibited by carbon monoxide just as that of the more strongly respiring cells is. If, in these experiments, oxygen tensions of less than one-tenth atmosphere are used, the oxygen of the oxyhaemoglobin in all the vessels must be displaced by carbon monoxide, otherwise errors due to the dissociation of oxyhaemoglobin are encountered.

All these precautions are unnecessary with the more strongly respiring blood cells, because so little material is needed that the formic acid formation from the carbon monoxide, and the dissociation of the oxyhaemoglobin are so small as to be of no significance in comparison with the respiration.

The experiments with red blood cells respiring at different levels show why cells which respire strongly are better experimental material than the feebly respiring cells. This is the reason why the action of carbon monoxide on respiration was discovered with the strongly respiring cells of baker's yeast.

9. Muscle

Of all experimental material, muscle is the most unsuitable for manometric experiments. If it is cut into thin pieces one is really working with muscle brei in which the normal respiratory enzyme chain is broken up. If the muscle is not cut up, the difficulty of diffusion especially at low oxygen tensions is too

† O. Warburg *et al.*, *Bioch. Zeitschr.* **242**, 170 (1931).

great. It is, therefore, necessary when the action of carbon monoxide on the respiration of muscle has to be studied, to perfuse the muscle artificially. The perfusion fluid must then be put under vacuum, and the liberated gases analysed. Since there are technical difficulties in this, such experiments have not yet been carried out.

If washed muscle brei is extracted with phosphate and the extract precipitated with acetic acid, the precipitate contains the muscle iron system whilst the yellow enzymes remain in the mother liquor. With this precipitate, which can catalytically oxidize succinic acid to fumaric acid, and is therefore called succinic acid oxidase, it is possible to verify in simple manometric experiments how the oxygen transporting iron of the muscle behaves towards carbon monoxide.

M. Dixon† in 1927 carried out such experiments, and as a result, he believed that succinic acid oxidase is not inhibited by carbon monoxide. Actually, succinic acid oxidase, just like the respiration of yeast, is inhibited, and the inhibition is light-sensitive as with yeast respiration. I would conclude from this that the respiration of the intact muscle is also inhibited by carbon monoxide.

It should be mentioned here, however, that according to the experiments of W. O. Fenn,‡ the respiration of intact muscle is not inhibited by carbon monoxide. Fenn found that carbon monoxide brought about an increase in the respiration. We cannot bring our own experiments into harmony with Fenn's results.

10. Alcoholic fermentation

When the fermentation of yeast cells in nitrogen is compared with that in carbon monoxide, oxygen being excluded in both cases, no difference is observed. For example we found:§

	In nitrogen at atmospheric pressure	*In carbon monoxide at atmospheric pressure*
Fermentation CO_2 in 30 min.	95 c.mm.	94 c.mm.

† M. Dixon, *Bioch. Journ.* **21**, 1211 (1927).

‡ W. O. Fenn and D. M. Cobb, *Amer. Journ. Physiology*, **102**, 379, 393 (1932).

§ O. Warburg, *Bioch. Zeitschr.* **177**, 471 (1926).

Since it was possible that the carbon monoxide pressure of 1 atmosphere might not have been sufficient to inhibit the fermentation, we increased the pressure to 60 atmospheres. The yeast cells were shaken in steel bottles with phosphate solution containing sugar, nitrogen being present in one case, carbon monoxide in the other. The yeast was finally centrifuged off, and the decrease in the sugar content of the solutions determined polarimetrically. In both cases exactly the same amount of sugar had disappeared. Calculated on the basis of carbon dioxide production, a normal Q value for the fermentation was obtained in both cases.

i.e. at 22° $Q_{CO_2}^{N_2} = 205$ c.mm. CO_2/mg. yeast per hour.

Alcoholic fermentation is, therefore, not inhibited by carbon monoxide. The fact that it is specifically inhibited by cyanide and nitric oxide is not incompatible with this result.†

11. Lactic acid fermentation

Since the components of the enzyme systems of lactic acid and alcoholic fermentation are the same up to carboxylase, it was to be expected from the behaviour of alcoholic fermentation that lactic acid fermentation would likewise not be inhibited by carbon monoxide. We found no inhibition of lactic acid fermentation using lactic acid bacteria, tumours, and red blood cells.

There are, however, lactic acid bacteria, the fermentation of which is not a pure lactic acid fermentation, but which produces hydrogen and carbon dioxide as well. In such bacteria the production of hydrogen and carbon dioxide is inhibited by carbon monoxide.

12. Butyric acid fermentation

When *Bacterium coli* is cultured in media containing formic acid, it acquires the capacity to ferment formic acid to carbon dioxide and hydrogen:

$$HCOOH = CO_2 + H_2.$$

† O. Warburg, *Bioch. Zeitschr.* **189**, 354 (1927).

This fermentation is inhibited by carbon monoxide as Stephenson and Stickland† found in 1932. In 1933 W. Kempner‡ discovered that the butyric acid fermentation in which hydrogen is likewise produced is inhibited by carbon monoxide.

$$C_6H_{12}O_6 = C_4H_8O_2 + 2CO_2 + 2H_2.$$

For example, the inhibition of gas formation with *Clostridium butyricum* amounted to:

In 5 vols. % CO	*In 25 vols.* % CO	*In 100 vols.* % CO
55%	82%	92%

When butyric acid bacteria, the respiration of which has been inhibited by carbon monoxide, are exposed to light intensities which appreciably lessen the carbon monoxide inhibition of aerobic respiration, as for example in yeast, the light has no action. Intensities a hundred times greater, however, also decrease the carbon monoxide inhibition of butyric acid fermentation.§ The gas-forming enzyme of butyric bacteria which reacts with the carbon monoxide is, therefore, an iron compound. The determination of its absorption spectrum is described in Chapter XVIII.

Kubowitz‖ found that with butyric acid bacteria in which the gas formation has been inhibited by carbon monoxide, the breakdown of the sugar continues at almost the same rate. Instead of the usual product of butyric acid fermentation, however, lactic acid is formed. Probably in butyric acid fermentation the course of the reaction is the same as for lactic acid fermentation up to the point where pyruvic acid is formed. When the gas-forming iron enzyme is blocked by carbon monoxide, the pyruvic acid, as in ordinary lactic acid fermentation, is reduced by the dihydropyridine to lactic acid. When the iron is free the pyruvic acid is decomposed according to the equation

$$CH_3COCOOH + H_2O = CH_3COOH + CO_2 + H_2.$$

The dihydropyridine does not reduce the pyruvic acid to lactic

† M. Stephenson and L. H. Stickland, *Biochem. Journ.* **26**, 712 (1932).
‡ W. Kempner, *Bioch. Zeitschr.* **257**, 41 (1933).
§ W. Kempner and F. Kubowitz, ibid. **265**, 245 (1933).
‖ F. Kubowitz, ibid. **274**, 285 (1934).

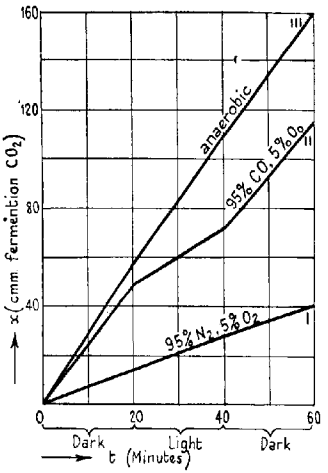


FIG. 7. Alcoholic fermentation by yeast.

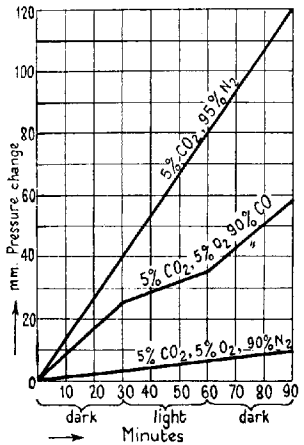


FIG. 8. Lactic acid fermentation by blood platelets.

acid, but instead the acetic acid is reduced to acetaldehyde, two molecules of which condense to form butyric acid:

$$2(CH_3CHO) = C_4H_8O_2.$$

13. Aerobic fermentation

Although the enzymes of alcoholic and lactic acid fermentation do not react with carbon monoxide, these fermentations can, however, be effected with carbon monoxide in a roundabout way through the oxygen transporting enzyme. If the oxygen transporting iron in respiring cells is blocked by carbon monoxide, the rate of aerobic fermentation increases to that of the anaerobic fermentation although the cells are saturated with oxygen. On exposure to light, however, the fermentation decreases again to the aerobic rate (Figs. 7 and 8).†,‡

Experiments such as are illustrated in Figs. 7 and 8 prove the incorrectness of all theories in which the Pasteur effect is explained by a direct action of oxygen on the fermentation enzymes. It can be no accident that carbon monoxide and light act on the fermentation under just those conditions of pressure and intensity at which the oxygen transporting iron adds on and splits off carbon monoxide. But it is still impossible to say how the oxygen-transporting enzyme causes the Pasteur effect; whether the ferric iron oxidizes the fermentation enzymes and thereby inactivates them (zymohexase comes to mind in this connexion), or whether other components in the respiratory enzyme chain inactivate the fermentation enzymes by oxidizing them, or whether the fermentation is inhibited by intermediate products of the respiration.

F. Lynen§ believes, and has tried to prove it experimentally, that respiration reduces the amount of free phosphate available for fermentation. If this is so, it is difficult to understand how it can be possible to arrange conditions‖ under which both processes, respiration and fermentation, can proceed simultaneously at their full rate.

† O. Warburg, *Bioch. Zeitschr.* **177,** 471 (1926); **189,** 354 (1927).
‡ A. Fujita, ibid. **197,** 189 (1928).
§ F. Lynen, *Liebigs Ann.* **546,** 120 (1941).
‖ O. Warburg, *Bioch. Zeitschr.* **172,** 432 (1926).

CHAPTER X

THE HAEM MODEL AND THE FERROUS CYSTEINE MODEL

If yeast cells, the respiration of which has been inhibited by carbon monoxide, are exposed to light of different colours, but of the same intensity, and the effect is expressed graphically as a function of the wave-length,† then a curve is obtained which we have called the 'action spectrum'. If every quantum of light absorbed, independent of the wave-length, produced the same effect, the 'action spectrum' would be an absorption spectrum of the carbon monoxide compound of the oxygen transporting enzyme.

We tackled the problem first using model experiments. The action spectra which were obtained by exposing to light biologically important carbon monoxide-iron compounds were compared with their absorption spectra, to find out if they were the same.

1. Haemin catalysis

Pure cysteine, free from heavy metals, is not oxidized in neutral solution by molecular oxygen. If a small amount of blood haemin is added, however, the haemin‡ transports oxygen to the cysteine by virtue of the valency change ferrous → ferric, until the cysteine has been oxidized to cystine. The catalysis is inhibited by carbon monoxide§ and the inhibition is light-sensitive.

This catalysis was not, however, a suitable model. Firstly, the catalytic activity of the haemin is so small that haemin concentrations which are too great for optical experiments are required. Secondly, the light sensitivity of the carbon monoxide inhibition is so small that the light intensities required for photochemical experiments are too great.

† O. Warburg and F. Negelein, *Bioch. Zeitschr.* **193**, 339 (1928).
‡ D. C. Harrison, *Biochem. Journ.* **18**, 1009 (1924).
§ H. A. Krebs, *Bioch. Zeitschr.* **193**, 347 (1928).

2. Nicotine haemochromogen, and pyridine haemochromogen

Anson and Mirsky† showed that in the biologically important haem compounds, the haems are combined with nitrogenous bases. H. A. Krebs‡ examined the influence of such bases on the haem catalysis of cysteine oxidation and found that nicotine haemochromogen and pyridine haemochromogen were much more active than the free haem. Whilst 1 mg. of iron in the free haem at 20° transported about 10,000 c.mm. oxygen per hour, this value increased on addition of nicotine or pyridine to about 1,000,000. The iron in the oxygen transporting enzyme is about 3,000 times more active than the latter,§ but the catalytic activity of nicotine or pyridine haemochromogen was good enough for our experiments.

Nicotine haemochromogen and pyridine haemochromogen are only markedly catalytically active in cysteine solutions if iron salts are added. It is not, therefore, the HS – group of the cysteine, but the iron of the ferrous cysteine which reacts with the haem iron. Therefore the rate of the catalysis is not proportional to the concentration of cysteine, but rather to the concentration of iron salts as long as the cysteine is in excess over the iron salts.

As we have two iron atoms acting in conjunction,

$$O_2 \rightarrow \text{haem iron} \rightarrow \text{cysteine iron} \rightarrow \text{cysteine},$$

suitable concentrations must be chosen so that the more direct route

$$O_2 \rightarrow \text{cysteine iron} \rightarrow \text{cysteine}$$

can be disregarded in favour of the route which takes in the haem iron. Complications could also arise in the inhibition of the catalysis by carbon monoxide and cyanide on account of the cysteine iron. In the inhibition experiments the concentrations can also be so chosen that the action of the inhibitors on the cysteine iron can be disregarded.

† M. L. Anson and A. E. Mirsky, *Journ. Physiology*, **60**, 50, 161, 221 (1925).
‡ H. A. Krebs, *Bioch. Zeitschr.* **204**, 322 (1929).
§ O. Warburg and F. Kubowitz, ibid. **214**, 5 (1929).

3. Action of carbon monoxide†

Carbon monoxide inhibits this catalysis and light causes the inhibition to disappear. If one plots the oxygen uptake as a function of time, curves analogous to those for the carbon monoxide inhibition of respiring cells are obtained (Figs. 9 and 10).

These carbon monoxide inhibitions are just as light-sensitive

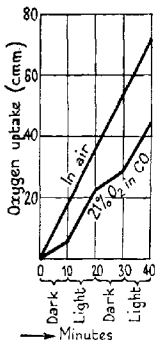


FIG. 9. Inhibition of the pyridine haemochromogen catalysis by carbon monoxide and the action of light.

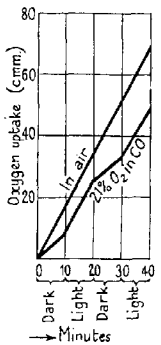


FIG. 10. Inhibition of nicotine haemochromogen catalysis by carbon monoxide and the action of light.

as those in cell respiration, therefore the pyridine and nicotine haemochromogen catalyses were very suitable for our optical model experiments.

4. Action of hydrogen cyanide‡

Cyanide inhibits the nicotine haemochromogen and the pyridine haemochromogen catalyses, and the action is reversible as in the case of cell respiration. Hydrogen cyanide therefore forms with both catalysts dissociable chemical compounds.

The important question as to which oxidation level of the iron reacts with the cyanide has been ascertained in the following way. If increasing amounts of cyanide are added to the

† H. A. Krebs, *Bioch. Zeitschr.* **204**, 322 (1929).
‡ Id., ibid. **189**, 354 (1927).

system in which pyridine haemochromogen transports the oxygen, half inhibition of the catalysis is found to take place at a cyanide concentration of $2{\cdot}60 \times 10^{-5}$ mole/litre.

If, on the other hand, increasing amounts of cyanide are added to pure solutions of the catalyst it is found that at a cyanide concentration of $2{\cdot}67 \times 10^{-5}$ mole/litre, half of the iron is combined with cyanide when the catalyst is in the ferric state. At the same cyanide concentration no iron is combined with the cyanide if the catalyst is in the ferrous state.

The cyanide reacts, therefore, with the ferric state and not with the ferrous state of the catalyst when inhibition takes place. Just as *in vivo* cyanide inhibits, in model experiments, not the oxidation of the ferrous iron but the reduction of the ferric iron. Perhaps the simple reaction†

$$\rangle\text{FeOH} + \text{HCN} = \rangle\text{FeCN} + \text{H}_2\text{O}$$

is responsible for the cyanide action in this case as well as *in vivo*.

5. Action spectrum‡

The same amount of a solution containing cysteine as substrate with ferrous sulphate and nicotine haemochromogen as catalysts was added to two manometric vessels of the same size. The vessels were then connected to a differential manometer. Gas spaces in both vessels were filled with the same mixture, 21 per cent. by volume of oxygen and 79 per cent. by volume of carbon monoxide. On shaking in a darkened thermostat no difference in the level of the differential manometer was observed because in both vessels the same amount of oxygen was used. The amount of oxygen used was small on account of the inhibition of the oxygen transport by the carbon monoxide. When, however, one of the vessels was exposed to light a difference in level occurred, because in the exposed vessel carbon monoxide was split off from the catalyst.

The light used was monochromatic and of measured intensity. From the intensity quanta and the manometer reading the

† O. Warburg, *Zeitschrift f. angewandte Chemie*, **45**, 1 (1932).
‡ O. Warburg and E. Negelein, *Bioch. Zeitschr.* **200**, 414 (1928).

action spectrum was worked out. The action spectrum was compared with the absorption spectrum of the catalyst, or rather the spectrum of the carbon monoxide compound, since it is the carbon monoxide compound of the catalyst which is affected by the light. The absorption spectrum was determined bolometrically for the same wave-lengths as had been used in

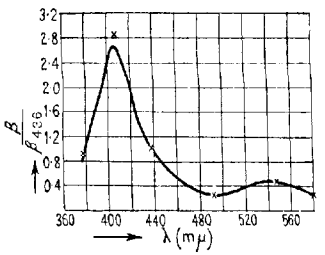


FIG. 11. Spectrum of the carbon monoxide nicotine haemochromogen. The line represents the bolometrically measured spectrum; the crosses, the points calculated from the photochemical action.

the determination of the action spectrum. Taking arbitrary values of 1 for the light absorption coefficient and the photochemical action for a wave-length of 436 mμ we found:

Wave-length mμ	*Photochemical action*	*Light absorption coefficient*
366	0·98	0·80
405	2·84	2·64
436	1·00	1·00
492	0·29	0·27
546	0·49	0·48
578	0·26	0·33

In Fig. 11 the result is expressed graphically. The line was obtained by joining the six points determined for the absorption spectrum. It is not a complete spectrum because the number of points taken was too small. The crosses are the points obtained from the photochemical action determinations.

Considering the limits of error in the measurement we can say that the action spectrum is really the absorption spectrum of the carbon monoxide compound of the catalyst.

6. Ferrocysteine

Our second model for testing the relationship between the action of the light and its absorption was ferrocysteine, the carbon monoxide compound† of which is decomposed according to the equation

$$Fe(CO)_2 \rightleftharpoons Fe + 2CO.$$

If a carbon monoxide ferrocysteine solution is exposed to light of constant intensity in a closed vessel, the carbon monoxide pressure increases until just as much carbon monoxide reacts per minute as is split off photochemically.

Optically thin layers of a carbon monoxide ferrocysteine solution were exposed to monochromatic light of different wave-lengths, the intensities being so equalized that all wave-lengths produced the same equilibrium pressure of carbon monoxide. From the intensity quanta corresponding to the same degree of action, the action spectrum was then worked out.

We compared the absorption spectrum measured bolometrically for the same wave-lengths with the photochemical action spectrum.

Assuming an arbitrary value of 1 for the light absorption coefficient and the photochemical action at wave-length 436 mμ the results were.‡

Wave-length mμ	*Photochemical action*	*Light absorption coefficient*
366	1·41	1·40
405	0·92	0·96
436	1·00	1·00
492	1·44	1·36
546	0·32	0·35
578	0·056	0·067

In Fig. 12 the result is graphically illustrated. Within the error of determination the action spectrum is the same as the absorption spectrum of the carbon monoxide compound.

† W. Cremer, *Bioch. Zeitschr.* **194**, 231 (1928); **206**, 228 (1929).
‡ O. Warburg and E. Negelein, ibid. **200**, 414 (1928).

7. Results

The expression

Action spectrum = Absorption spectrum

has been proved to apply for two iron compounds both of which are present in the living cell, but which are quite different in chemical constitution. Moreover, the iron in one compound is

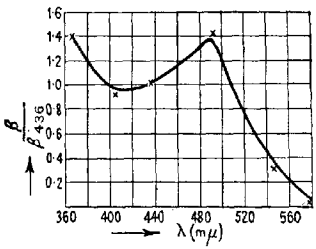


FIG. 12. Spectrum of carbon monoxide-ferrocysteine. The line represents the bolometrically measured spectrum; the crosses, the points calculated from the photochemical action.

combined with one molecule, and in the other with two molecules of carbon monoxide.

Further, the methods by which the action of light was determined were different. In the case of the haem model the extent of the light action was determined, not by the amount of carbon monoxide removed from the iron, but by a reaction dependent on this, namely, the increase in catalytic oxygen transfer by the iron set free on exposure. In the case of the ferrocysteine model, the amount of carbon monoxide split off from the iron was used as a measure of the action of the light.

The two methods are supplementary to one another. The first method is the one which has been used with cell respiration and the aim of the model experiment is fully realized. The second method depends on the determination of the primary product of the photochemical action and is, therefore, more comprehensive in a physical sense.

On the basis of these model experiments alone the expression

Action spectrum = Absorption spectrum

could be applied directly to the carbon monoxide inhibition of cell respiration, i.e. to the carbon monoxide compound of the oxygen transporting enzyme. Further theoretical and experimental investigation has, however, been necessary because the action spectrum gives only the relative absorption spectrum, whilst for chemical classification of the enzyme it is desirable to determine also the molar absorption. In contrast to the spectrum analysis of an element the position of the absorption bands is not sufficient for identification in the case of carbon compounds.

CHAPTER XI

PHOTOCHEMICAL DECOMPOSITION CONSTANT

WHEN cells, the respiration of which has been inhibited by carbon monoxide, are exposed to light, there is a small though definite time lag till the respiration value in darkness increases to that in light, the latter corresponding to the colour and the intensity of the light used. It can be readily understood that this transition time for a given wave-length and intensity will be the shorter the greater the absorption of the light by the carbon monoxide compound of the oxygen transporting enzyme. This relationship can be used to determine the light absorption coefficient from the transition times in monochromatic light.

In this chapter the formulae necessary for the calculation will be derived, first, for the simpler cases, then for the oxygen transporting enzyme. I have chosen as the heading of this chapter 'Photochemical Decomposition Constant', because this subject is the main one discussed and its experimental determination is dealt with.

1. Photochemical yield

When p light quanta are absorbed by a substance, and as a result p' molecules of the substance react chemically, the photochemical yield

$$\phi = \frac{p'}{p}. \tag{1}$$

If $p' = p$ then $\phi = 1$. This is Einstein's photochemical equivalence law.

2. Quantum intensity

If the energy of the incident light is i' gram calories per sq. cm. per minute, the quantum intensity i is given by

$$i = \frac{i'}{N_0 h\nu} [\text{mole quanta/cm.}^2\text{/minute}],$$

where $N_0 h\nu$ is the energy of 1 mole quantum in calories. This

energy is proportional to the frequency or inversely proportional to the wave-length of the light and it amounts to:

Wave-length ($m\mu$)	$N_0 h\nu$ (*cal./mole*)
366	77 500
405	70 050
436	65 100
492	57 700
546	52 000
578	49 100
660	43 000
1 000	28 400

3. Photochemical decomposition constant

In a solution exposed to light let

c = concentration of the light sensitive substance $\left[\frac{\text{mole}}{\text{cm.}^3}\right]$

β = its light absorption coefficient $\left[\frac{\text{cm.}^2}{\text{mole}}\right]$

i = the quantum intensity $\left[\frac{\text{mole quanta}}{\text{cm.}^2\ \text{min.}}\right]$

d = the thickness of the solution layer (cm.)

q = the surface exposed (cm.^{2})

t = time of exposure (minutes)

ϕ = photochemical yield $\left[\frac{\text{mole substance}}{\text{mole quanta}}\right]$.

Let the light be monochromatic, and the concentration c and the thickness of the layer d be so small that only a very small part of the light is absorbed as it passes through the solution. Thus, the light intensities of the incident and the issuing light may be assumed to be the same (Fig. 13). Then in the exposed solution

$$i\beta cdq$$

mole quanta are absorbed per minute.

1 mole of the substance reacts per minute when the photochemical yield = 1, and a fraction ϕ when the photochemical yield equals ϕ. Therefore, in the solution there are

$$-\frac{dc}{dt}qd = \phi i\beta qdc \qquad (2)$$

moles substance per minute decomposed.

If we divide both sides of (2) by the volume of the solution qd, we obtain the velocity of the photochemical reaction for the quantum intensity i:

$$-\frac{dc}{dt} = \phi i \beta c. \tag{3}$$

If we divide both sides of (3) by the concentration c, we obtain

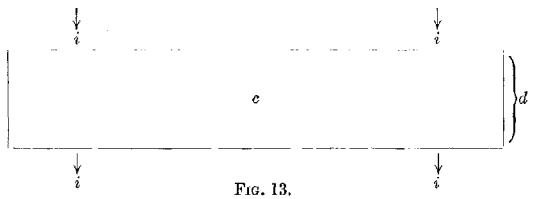


FIG. 13.

the photochemical decomposition constant for the quantum intensity i:

$$\frac{-dc/dt}{c} = \phi i \beta. \tag{4}$$

To simplify let $$\left(\frac{-dc/dt}{c}\right) = z_i,$$

then instead of (4) we can write

$$z_i = \phi i \beta. \tag{5}$$

The meaning of this remarkably simple equation which I derived in 1928† is clear. If it is desired to determine in any photochemical reaction the time required for half decomposition to take place, and if the light intensity which brings about the decomposition is known, then equation (5) gives the product $\phi\beta$

$$\phi\beta = \frac{z_i}{i},$$

that is, the photochemical yield ϕ if the light absorption coefficient β is known, or β if ϕ is known.

4. Irreversible photochemical reaction

The determination of the photochemical decomposition constant is simple if the products of the decomposition do not react

† O. Warburg and E. Negelein, *Bioch. Zeitschr.* **202**, 202 (1928) [p. 216].

again with one another. As an example of this we may take the destruction of the enzyme urease by ultra-violet light.†

Very dilute solutions of the enzyme were exposed to ultra-violet light until a part of the enzyme was destroyed, that is, made incapable of converting urea to carbon dioxide and ammonia.

If the activity of the enzyme was W_0 before, and W after the exposure, the photochemical decomposition constant for an exposure of t minutes with a quantum intensity i would be

$$z_i = \int_{W_0}^{W} \frac{-dW/dt}{W} = \frac{1}{t} \ln \frac{W_0}{W}. \tag{6}$$

A necessary condition of such experiments is that the exposed solution should be optically thin. Only then is the photochemical decomposition constant the same throughout the solution, and only then is it immaterial whether or not the products of the decomposition absorb light.

5. Reversible photochemical reaction

Carbon monoxide haemochromogens dissociate in the dark according to the equation

$$\mathrm{FeCO} \rightleftharpoons \mathrm{Fe} + \mathrm{CO}. \tag{7}$$

On exposure to light, in addition to the dark reaction there is a photochemical decomposition of the carbon monoxide compound, whilst the reverse reaction of the decomposition products is not affected by the light. In a solution of a carbon monoxide haemochromogen exposed to light we therefore have three simultaneous reactions, two dark reactions and a photochemical one. The problem is to determine in such solutions the photochemical decomposition constant. Let

Fe = the concentration of the haemochromogen,
FeCO = the concentration of the CO-haemochromogen,
z_d = the dark decomposition constant of the FeCO,
z_i = the photochemical decomposition constant of the FeCO,
i = the light intensity,

† F. Kubowitz and E. Haas, ibid. **257**, 337 (1933).

b = the velocity constant of the reverse reaction,

p_{CO} = the pressure of the CO,

t = the time.

Then,

$$\text{in the dark: } \frac{d\text{Fe}}{dt} = z_d\,\text{FeCO} - bp_{CO}\text{Fe}, \tag{8}$$

$$\text{in the light: } \frac{d\text{Fe}}{dt} = (z_d+z_i)\text{FeCO} - bp_{CO}\text{Fe}, \tag{8a}$$

and at equilibrium, when $d\text{Fe}/dt = 0$, we have

$$\text{in the dark: } \frac{\text{Fe}p_{CO}}{\text{FeCO}} = \frac{z_d}{b}, \tag{9}$$

$$\text{in the light: } \frac{\text{Fe}p_{CO}}{\text{FeCO}} = \frac{z_d+z_i}{b}. \tag{9a}$$

In equations (8) and (8a) the carbon monoxide pressure p_{CO} should remain constant at all times, a condition which can easily be fulfilled experimentally. Then bp_{CO} is a velocity constant independent of time.

Let $$bp_{CO} = \rho, \quad z_d = \omega_d, \quad z_d+z_i = \omega_h. \tag{10}$$

Dividing both sides of equations (8) and (8a) by $\sum$Fe and letting

$$\frac{\text{Fe}}{\sum\text{Fe}} = n, \qquad \frac{\text{FeCO}}{\sum\text{Fe}} = 1-n,$$

we obtain (8) and (8a) in the form

$$\text{darkness: } \frac{dn}{dt} = \omega_d(1-n) - \rho n, \tag{11}$$

$$\text{light: } \frac{dn}{dt} = \omega_h(1-n) - \rho n. \tag{11a}$$

At equilibrium ($dn/dt = 0$), n in darkness has the value n_d and in light the value n_h determined by the light intensity. These equilibrium values of n are, according to (11) and (11a), related to the velocity constants ω and ρ according to the expressions:

$$\frac{\omega_d}{\rho} = \frac{n_d}{1-n_d}, \qquad \frac{\omega_d}{\omega_d+\rho} = n_d, \tag{12}$$

$$\frac{\omega_h}{\rho} = \frac{n_h}{1-n_h}, \qquad \frac{\omega_h}{\omega_h+\rho} = n_h. \tag{12a}$$

When the reaction is not at equilibrium n changes with time. When we go from dark equilibrium and expose, n increases from n_d to n_h. When we go from light equilibrium and darken, n decreases from n_h to n_d. These changes of n during the transition periods we can obtain as a function of the time t by solving the differential equations (11) and (11a). Using (12) the solutions are

$$\text{darkness to light: } n = n_d + (n_0 - n_d)e^{-(\omega_d+\rho)t}, \tag{13}$$

$$\text{light to darkness: } n = n_h + (n_0 - n_h)e^{-(\omega_h+\rho)t} \tag{13a}$$

where $n_0 = n$ when $t = 0$.

If n can be measured as a function of the time t, then with the help of equations (13) the velocity constants $(\omega+\rho)$ can be determined, since from (13) and (13a) we have

$$\text{dark to light: } (\omega_d+\rho) = \frac{1}{t}\ln\frac{n_0-n_d}{n-n_d}, \tag{14}$$

$$\text{light to dark: } (\omega_h+\rho) = \frac{1}{t}\ln\frac{n_0-n_h}{n-n_h}. \tag{14a}$$

Our problem is, therefore, theoretically solved since according to definition (10) the photochemical decomposition constant

$$z_i = (\omega_h+\rho)-(\omega_d+\rho). \tag{15}$$

Bücher and Negelein tested these equations which I derived in 1928† with solutions of carbon monoxide-pyridine haemochromogen.‡ They found that $(\omega_h+\rho)$ and $(\omega_d+\rho)$ were constant for the whole course of the transition times dark → light, and light → dark, also that the difference between the two constants was proportional to the light intensity as required by theory.

6. Carbonylhaemoglobin

If carbon monoxide haemochromogen is a model for the simplest photochemical dissociation, then carbonylhaemoglobin in the presence of oxygen is the model of the photochemical displacement:

$$FeCO + O_2 \rightleftharpoons FeO_2 + CO.$$

† O. Warburg and E. Negelein, *Bioch. Zeitschr.* **202,** 202 (1928).
‡ Th. Bücher and E. Negelein, ibid. **311,** 163 (1942).

The theoretical treatment of this model is more complicated since there are not three but five reactions, namely, four dark reactions and one photochemical reaction. The formulae for the determination of the photochemical decomposition constants are obtained as follows:

In a haemoglobin solution containing carbon monoxide and oxygen we have in the dark the reversible reactions

$$\mathrm{FeCO} \rightleftharpoons \mathrm{Fe} + \mathrm{CO},$$

$$\mathrm{FeO_2} \rightleftharpoons \mathrm{Fe} + \mathrm{O_2},$$

the velocities of which are

$$-\frac{d(\mathrm{FeCO})}{dt} = z(\mathrm{FeCO}) - bp_{\mathrm{CO}}\mathrm{Fe}, \tag{16}$$

$$-\frac{d(\mathrm{FeO_2})}{dt} = Z(\mathrm{FeO_2}) - Bp_{\mathrm{O_2}}\mathrm{Fe}, \tag{17}$$

where z and b and Z and B are the velocity constants.

The pressures of oxygen and of carbon monoxide are so great that Fe can always be disregarded in comparison with FeO_2 and FeCO. Then at any time the decrease in FeCO = the increase in FeO_2, therefore, from (16) and (17),

$$z(\mathrm{FeCO}) - bp_{\mathrm{CO}}\mathrm{Fe} = -Z(\mathrm{FeO_2}) + Bp_{\mathrm{O_2}}\mathrm{Fe}. \tag{18}$$

Solving equation (18) for Fe, we have

$$\mathrm{Fe} = \frac{z(\mathrm{FeCO}) + Z(\mathrm{FeO_2})}{bp_{\mathrm{CO}} + Bp_{\mathrm{O_2}}}; \tag{19}$$

substituting (19) in (17) we obtain

$$\frac{d(\mathrm{FeO_2})}{dt} = \left[z\frac{Bp_{\mathrm{O_2}}}{Bp_{\mathrm{O_2}} + bp_{\mathrm{CO}}}\right]\mathrm{FeCO} - \left[Z\frac{bp_{\mathrm{CO}}}{bp_{\mathrm{CO}} + Bp_{\mathrm{O_2}}}\right]\mathrm{FeO_2}, \tag{20}$$

and at equilibrium, when $d(\mathrm{FeO_2})/dt = 0$,

$$\frac{\mathrm{FeO_2}}{\mathrm{FeCO}}\frac{p_{\mathrm{CO}}}{p_{\mathrm{O_2}}} = \frac{z}{b}\frac{B}{Z} = K, \tag{21}$$

where K is the equilibrium constant for the partition reaction.

Actually for the application of equation (20) the simple condition which is always easy to fulfil experimentally, namely, that the pressure of the oxygen and the carbon monoxide should

not change during the course of the reaction, must be realized. Then the bracketed expressions in (20) may be treated as constant. Further, in order to apply equation (20), bp_{CO} should be small in comparison with Bp_{O_2}. According to Glen Millikan†‡ B is about 30 times as great as b; the oxygen pressure p_{O_2} is, however, in all applications of equation (20) considerably greater than the carbon monoxide pressure p_{CO}. Taking this into consideration the bracketed expressions in equation (20) become

$$\left[z\frac{Bp_{O_2}}{Bp_{O_2}+bp_{CO}}\right]=z \quad \text{and} \quad \left[Z\frac{bp_{CO}}{bp_{CO}+Bp_{O_2}}\right]=Z\frac{bp_{CO}}{Bp_{O_2}},$$

and instead of (20) we can write

$$\frac{d(FeO_2)}{dt}=z(FeCO)-Z\frac{bp_{CO}}{Bp_{O_2}}(FeO_2). \tag{22}$$

Further, substituting

$$z=\omega \quad \text{and} \quad Z\frac{bp_{CO}}{Bp_{O_2}}=\rho, \tag{23}$$

(22) becomes $$\frac{d(FeO_2)}{dt}=\omega(FeCO)-\rho(FeO_2). \tag{24}$$

If we divide both sides of (24) by $\sum$ Fe and put

$$\frac{FeO_2}{\sum Fe}=n \quad \text{and} \quad \frac{FeCO}{\sum Fe}=1-n,$$

we obtain (24) as $$\frac{dn}{dt}=\omega(1-n)-\rho n. \tag{25), like (11)}$$

The mathematical treatment of the simple FeCO dissociation and the displacement of the CO by O_2 leads, therefore, to the same differential equation. The physical meaning of the variable n and the constant ρ is different in the two cases, but the meaning of the constant ω is, and this is most important, in both cases the same. ω is in both cases the decomposition constant of the FeCO.

Further development involves the repetition of the operations (11) to (15).

† Glen A. Millikan, *Proc. Royal Soc. London*, B **120**, 336 (1936).
‡ Id., *Physiological Reviews*, **19**, 503 (1939) [Baltimore U.S.A.].

Dividing (25) for light and darkness gives:

$$\text{dark to light: } \frac{dn}{dt} = \omega'_d(1-n)-\rho n, \tag{26}$$

$$\text{light to darkness: } \frac{dn}{dt} = \omega_h(1-n)-\rho n. \tag{26a}$$

Solving the differential equations for n gives:

$$\text{dark to light: } n = n_d+(n_0-n_d)e^{-(\omega_d+\rho)t}, \tag{27}$$

$$\text{light to dark: } n = n_h+(n_0-n_h)e^{-(\omega_h+\rho)t}, \tag{27a}$$

where $n_0 = n$ when $t = 0$.

Solving for $\omega+\rho$ gives

$$\text{dark to light: } (\omega_d+\rho) = \frac{1}{t}\ln\frac{n_0-n_d}{n-n_d}, \tag{28}$$

$$\text{light to dark: } (\omega_h+\rho) = \frac{1}{t}\ln\frac{n_0-n_h}{n-n_h}, \tag{28a}$$

and the difference between the two velocity constants gives the photochemical decomposition constant of the carbonylhaemoglobin z_i,

$$z_i = (\omega_h+\rho)-(\omega_d+\rho). \tag{29}$$

The equations developed in 1928 were tested in 1942 by Bücher and Negelein with haemoglobin solutions.† They found that $(\omega_d+\rho)$ and $(\omega_h+\rho)$ were constant for the whole course of the transition periods dark → light and light → dark, and the difference between the two constants was proportional to the light intensity as required by theory.

7. Oxygen transporting enzyme‡

Since in living cells, the respiration of which is inhibited by carbon monoxide, oxygen and carbon monoxide compete for the iron of the oxygen transporting enzyme, the partition of the enzyme between oxygen and carbon monoxide can be treated in an analogous manner to that of the partition of haemoglobin. Mathematically it is immaterial whether the ferrous iron is oxygenated with oxygen to FeO_2 or whether it is oxidized to

† Th. Bücher and E. Negelein, *Bioch. Zeitschr.* **311**, 163 (1942).

‡ O. Warburg and E. Negelein, ibid. **202**, 202 (1928); **214**, 64 (1929).

ferric iron. It is also immaterial whether the ferrous iron in the haemoglobin is reformed by dissociation of FeO_2 or by reduction of ferric iron. The starting equations for the partition of the *enzyme are analogous to (16) and (17):*

$$-\frac{d(\mathrm{FeCO})}{dt} = z(\mathrm{FeCO}) - bp_{\mathrm{CO}}\mathrm{Fe}, \tag{30}$$

$$-\frac{d(\mathrm{Ferric})}{dt} = Z'\mathrm{Ferric} - Bp_{\mathrm{O_2}}\mathrm{Fe}, \tag{30a}$$

where z, b, and B have the same significance as in (16) and (17), whilst Z' is the velocity constant for the reduction of the ferric iron. Although it is probably incorrect to treat Z' as a constant at all the possible changing values for the ferric concentration, the success of the theory has justified the validity of this simple assumption which, moreover, at the time could not be replaced by any other.

The pressures of oxygen and carbon monoxide are so great that free Fe can always be disregarded in comparison with ferric iron and FeCO. We then continue from (30) and (30 *a*) as in the previous section:

$$\frac{d\,\mathrm{Ferric}}{dt} = \left[z\frac{Bp_{\mathrm{O_2}}}{Bp_{\mathrm{O_2}}+bp_{\mathrm{CO}}}\right]\mathrm{FeCO} - \left[Z'\frac{bp_{\mathrm{CO}}}{bp_{\mathrm{CO}}+Bp_{\mathrm{O_2}}}\right]\mathrm{Ferric}, \tag{31}$$

and at equilibrium, when $\frac{d\,\mathrm{Ferric}}{dt} = 0$,

$$\frac{\mathrm{Ferric}}{\mathrm{FeCO}}\frac{p_{\mathrm{CO}}}{p_{\mathrm{O_2}}} = \frac{z}{b}\frac{B}{Z'} = K, \tag{32}$$

where K is the equilibrium constant of the partition reaction.

It is essential for the application of (31) that the simplified condition, namely that the oxygen and the carbon monoxide pressure shall not change during the course of reaction, should apply. This is always realized experimentally. The bracketed expressions in (31) may then be taken as constant.

Also in the application of equation (31) bp_{CO} can be disregarded in comparison with $Bp_{\mathrm{O_2}}$.† Z' then amounts to at least 1,000

† O. Warburg and F. Kubowitz, ibid. **214**, 5 (1929).

reciprocal minutes whilst the expression

$$\left[Z' \frac{bp_{CO}}{bp_{CO}+Bp_{O_2}}\right]$$

is of the order of 1 reciprocal minute, which is only possible if the factor $\frac{bp_{CO}}{bp_{CO}+Bp_{O_2}}$ is very small.

Taking this into account, (31) becomes

$$\frac{d\,\text{Ferric}}{dt} = z(\text{FeCO}) - \left[Z' \frac{bp_{CO}}{Bp_{O_2}}\right]\text{Ferric}. \tag{33}$$

Substituting

$$\left.\begin{aligned} z = \omega \quad \text{and} \quad Z' \frac{bp_{CO}}{Bp_{O_2}} = \rho \\ \frac{\text{Ferric}}{\sum \text{Fe}} = n \quad \text{and} \quad \frac{\text{FeCO}}{\sum \text{Fe}} = 1-n \end{aligned}\right\}, \tag{34}$$

instead of (33) we can write

$$\frac{dn}{dt} = \omega(1-n) - \rho n.$$

(35), as for (25) and (11)

The mathematical treatment of the partition of the enzyme and of haemoglobin leads therefore to the same differential equation. The significance of the variable n and the constant ρ is different in the two cases, but the significance of the constant ω, and this is important, is the same in both cases. ω is in both cases the decomposition constant of the FeCO.

Dividing (35) as we did (25) into light and dark, we obtain

$$\text{dark:} \quad \frac{dn}{dt} = \omega_d(1-n) - \rho n, \tag{35}$$

$$\text{light:} \quad \frac{dn}{dt} = \omega_h(1-n) - \rho n, \tag{35a}$$

and solving both equations for n we obtain

$$\text{dark to light:} \quad n = n_d + (n_0 - n_d)e^{-(\omega_d+\rho)t}, \tag{36}$$

$$\text{light to dark:} \quad n = n_h + (n_0 - n_h)e^{-(\omega_h+\rho)t}, \tag{36a}$$

where $n_0 = n$ when $t = 0$.

If, in the case of the enzyme, one could measure in the

transition time t the number n as a function of t, one could calculate for the enzyme just as for haemoglobin, in accordance with equation (28), the velocity constants $\omega+\rho$ and from them the photochemical decomposition constant.

Experimentally, n for the enzyme is the respiration A divided by the uninhibited respiration A_0

$$n = \frac{\text{Ferric}}{\sum \text{Fe}} = \frac{A}{A_0}.$$

Since, however, each respiration measurement takes several minutes, and since the total transition time is of the order of one minute, it is technically impossible to determine the change of n with time in the transition periods. What one can measure, however, is the total value of the respiration as it continuously changes. This will be the integral

$$A_0 \int n\, dt.$$

How the velocity constants $(\omega+\rho)$ are calculated from this integral is shown in the next section.

8. Dark decomposition constant of the enzyme

If A_0 is the uninhibited respiration, then by definition nA_0 is the inhibited respiration. We multiply equation (36) by $A_0\,dt$ and we thus obtain the oxygen consumption for a small period of time dt:

$$\text{dark to light: } A_0 n\, dt = A_0 n_d\, dt + A_0(n_0 - n_d)e^{-(\omega_d+\rho)t}\, dt; \tag{37}$$

$$\text{light to dark: } A_0 n\, dt = A_0 n_h\, dt + A_0(n_0 - n_h)e^{-(\omega_h+\rho)t}\, dt. \tag{37a}$$

If we integrate from 0 to t we obtain the oxygen consumption in the time t

$$\text{dark to light: } A_0 \int_0^t n\, dt = A_0 n_d t + A_0 \frac{n_0 - n_d}{\omega_d+\rho}\left[1 - e^{-(\omega_d+\rho)t}\right]; \tag{38}$$

$$\text{light to dark: } A_0 \int_0^t n\, dt = A_0 n_h t + A_0 \frac{n_0 - n_h}{\omega_h+\rho}\left[1 - e^{-(\omega_h+\rho)t}\right]. \tag{38a}$$

n_0 in this equation is n for $t = 0$. If one starts from the light

equilibrium and goes to darkness, then $n_0 = n_h$. If one starts from the dark equilibrium and exposes to light then $n_0 = n_d$. If the oxygen consumption has been measured in the transition period, that is the integral $A_0 \int_0^t n\,dt$, and if A_0 and n_d and n_h are known, the velocity constants $(\omega+\rho)$ are the only unknowns in equation (38) from which, therefore, they can be calculated.

Since the measurement of the respiration during a transition period is too inaccurate on account of the shortness of the transition period, it is arranged that several light and dark periods follow one another, that is, the exposure is intermittent. Thus the transition effect can be repeated as often as required, and it can be measured more accurately than if it were limited to one transition period.

If for the intermittent exposure, the dark period is ϑ and the light period τ, the n values at the end of the dark and light periods can be called n_ϑ and n_τ. Then the dark period begins at n_τ and the light period at n_ϑ: therefore

$$\text{dark to light: } n_0 = n_\tau;$$
$$\text{light to dark: } n_0 = n_\vartheta.$$

If these n values are substituted in (38) and (38 a) we obtain

$$\text{dark to light: } A_0 \int_0^\vartheta n\,dt = A_0 n_d \vartheta + A_0 \frac{n_\tau - n_d}{\omega_d + \rho}\left[1 - e^{-(\omega_d+\rho)\vartheta}\right]; \tag{39}$$

$$\text{light to dark: } A_0 \int_0^\tau n\,dt = A_0 n_h \tau + A_0 \frac{n_\vartheta - n_h}{\omega_h + \rho}\left[1 - e^{-(\omega_h+\rho)\tau}\right]. \tag{39 a}$$

We shall not go over here the general theory for intermittent exposure but shall limit ourselves to the special case in which the light intensity in the light period is large. Then $\omega_h+\rho$ is large and the steady light value is reached so quickly that one has the steady light respiration during the whole light period. Then

$$\text{light to dark: } A_0 \int_0^\tau n\,dt = A_0 n_h \tau$$

and

$$n_\tau = n_h.$$

Substituting this in (39) and (39a) we obtain

$$\text{dark to light: } A_0 \int_0^{\vartheta} n\,dt = A_0 n_d \vartheta + A_0 \frac{n_h - n_d}{\omega_d + \rho}\left[1 - e^{-(\omega_d+\rho)\vartheta}\right]; \tag{40}$$

$$\text{light to dark: } A_0 \int_0^{\tau} n\,dt = A_0 n_h \tau. \tag{40a}$$

By adding together (40) and (40a) we obtain the oxygen consumption under intermittent exposure for the time $\vartheta + \tau$:

$$A_0 \int_0^{\tau} n\,dt + A_0 \int_0^{\vartheta} n\,dt = A_0 n_h \tau + A_0 n_d \vartheta + A_0 \frac{n_h - n_d}{\omega_d + \rho}\left[1 - e^{-(\omega_d+\rho)\vartheta}\right]. \tag{41}$$

From this equation the equilibrium constant $\omega_d + \rho$ can be calculated, if the oxygen consumption of a given cell suspension has been determined under the four following conditions:

1. In air to give A_0.
2. In 5 per cent. by volume O_2 and 95 per cent. by volume CO in the dark to give $A_0 n_d \vartheta$.
3. In 5 per cent. by volume O_2 and 95 per cent. by volume CO in the light to give $A_0 n_h \tau$.
4. In 5 per cent. by volume O_2 and 95 per cent. by volume CO intermittently to give

$$A_0 \int_0^{\tau} n\,dt + A_0 \int_0^{\vartheta} n\,dt.$$

If $(\omega_d + \rho)$ has been determined according to (41) (by test) then ω_d and ρ are known, since

$$\text{dark: } \frac{dn}{dt} = \omega_d(1-n) - \rho n; \tag{35}$$

and at equilibrium, when $n = n_d$,

$$0 = \omega_d(1-n_d) - \rho n_d, \qquad \frac{\omega_d}{\rho} = \frac{n_d}{1-n_d}, \tag{42}$$

therefore

$$\omega_d = n_d(\omega_d + \rho). \tag{43}$$

Thus one obtains from four respiration measurements the darkness decomposition constant ω_d of the carbon monoxide compound of the oxygen transporting enzyme.

Note. It should be mentioned that in the calculation of $(\omega_d+\rho)$ from equation (41) the intensity of the light need not be known. The light serves only to reach the steady light value. If this steady light value is nearly 1 the condition that the light intensity should be great is fulfilled. Then, according to (35*a*), when light equilibrium is reached

$$\frac{\omega_h}{\rho} = \frac{n_h}{1-n_h}, \tag{44}$$

and if ω_h is large in comparison with ρ then $(\omega_h+\rho)$ will also be large in comparison with $(\omega_d+\rho)$.

9. Example

We shall illustrate the application of equation (41) by taking the figures from the *Biochemische Zeitschrift*, vol. **214**, p. 91 (1929).

There we found for yeast cells at 10°:

Respiration in air $= A_0 = 0{\cdot}604$ c.mm. per minute,
n_d in 5 vol. per cent. O_2 and 95 vol. per cent. $CO = 0{\cdot}25$,
n_h in 5 vol. per cent. O_2 and 95 vol. per cent. $CO = 0{\cdot}97$.
Dark period $\vartheta = 0{\cdot}75$ minute.
Bright period $\tau = 0{\cdot}75$ minute.
Oxygen consumption in the time $3(\vartheta+\tau) = 2{\cdot}21$ c.mm.,
therefore in the time $(\vartheta+\tau) = 0{\cdot}74$ c.mm.

Substituting these values in (41) we obtain

$$0{\cdot}74 = 0{\cdot}604\times 0{\cdot}97\times 0{\cdot}75 + 0{\cdot}604\times 0{\cdot}25\times 0{\cdot}75 + \\ + 0{\cdot}604\times\frac{0{\cdot}72}{\omega_d+\rho}\times\left[1-e^{-(\omega_d+\rho)\times 0{\cdot}75}\right],$$

$$0{\cdot}74 = 0{\cdot}553+\frac{0{\cdot}435}{\omega_d+\rho}\left[1-e^{-(\omega_d+\rho)\times 0{\cdot}75}\right].$$

The right-hand side becomes

$0{\cdot}68$ with $(\omega_d+\rho) = 3{\cdot}0$,
$0{\cdot}72$ with $(\omega_d+\rho) = 2{\cdot}0$,
$0{\cdot}74$ with $(\omega_d+\rho) = 1{\cdot}6$,

$(\omega_d+\rho)$ is therefore about $1{\cdot}6\left[\frac{1}{\text{minutes}}\right]$ and, according to (43),

$$\omega_d = n_d(\omega_d+\rho) = 0{\cdot}250\times 1{\cdot}6 = 0{\cdot}4\left[\frac{1}{\text{minutes}}\right],$$

$$\rho = 1{\cdot}6-0{\cdot}4 = 1{\cdot}2\left[\frac{1}{\text{minutes}}\right],$$

and according to (44) approximating

$$\frac{\omega_h}{\rho} = \frac{n}{1-n_h} = \frac{0{\cdot}97}{0{\cdot}03},$$

$$\omega_h = \rho\frac{0{\cdot}97}{0{\cdot}03} = 1{\cdot}2\times 32{\cdot}4 = 38{\cdot}8\left[\frac{1}{\text{minutes}}\right],$$

$$\omega_h+\rho = 38{\cdot}8+1{\cdot}2 = 40\left[\frac{1}{\text{minutes}}\right].$$

It is instructive to calculate, using the above values for $(\omega_d+\rho)$ and $(\omega_h+\rho)$, how the number n changes with intermittent exposure during the course of the dark period and the light period. For this calculation we can use equations (36) and (36a) in which $n_0 = n_\tau$ for the dark period and $n_0 = n_\vartheta$ for the bright period:

$$\text{dark: } n = n_d+(n_\tau-n_d)e^{-(\omega_d+\rho)t}; \tag{36}$$

$$\text{light: } n = n_h+(n_\vartheta-n_h)e^{-(\omega_h+\rho)t}. \tag{36a}$$

For $n_h = 0{\cdot}97$; $n_d = 0{\cdot}25$; $(\omega_d+\rho) = 1{\cdot}6$; $(\omega_h+\rho) = 40$; and $\vartheta = \tau = 0{\cdot}75$ minutes, we obtain the values:

Dark		*Light*	
t (minutes)	n	t (minutes)	n
0	$0{\cdot}97 = n_\tau = n_h$	0	$0{\cdot}466 = n_\vartheta$
0·15	0·816	0·015	0·694
0·30	0·695	0·030	0·819
0·45	0·600	0·045	0·887
0·60	0·526	0·060	0·925
$\vartheta = 0{\cdot}75$	$0{\cdot}466 = n_\vartheta$	0·075	0·945
		0·15	$0{\cdot}97 = n_h$
		$\tau = 0{\cdot}75$	$0{\cdot}97 = n_\tau = n_h$

Whilst, therefore, at the end of the dark period the steady dark value is not reached, in the light period, after only 0·15 minutes, the steady light value n_h is attained. The graphic illustration in Fig. 14 makes this result clear.

10. Photochemical decomposition constant of the enzyme

In the equations

$$\text{dark: } \frac{dn}{dt} = \omega_d(1-n)-\rho n\,; \tag{35}$$

$$\text{light: } \frac{dn}{dt} = \omega_h(1-n)-\rho n\,; \tag{35a}$$

ω_d is by definition the darkness dissociation constant of the

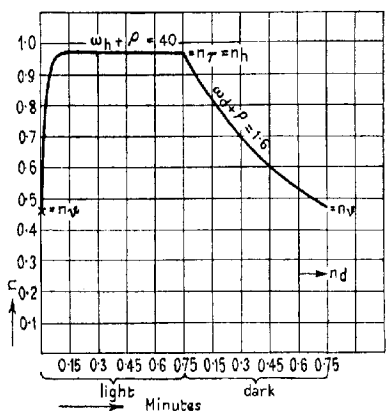


FIG. 14. n as a function of t with intermittent illumination. High intensity of light, i.e. $(\omega_h+\rho)$ large in comparison with $(\omega_d+\rho)$.

carbon monoxide compound of the enzyme. The light dissociation constant ω_h is the sum of the dark decomposition constant and the photochemical decomposition constant

$$\omega_d = z_d,$$
$$\omega_h = z_i+z_d;$$

hence $$\omega_h-\omega_d = z_i.$$

The determination of the photochemical decomposition constant z_i requires therefore a knowledge of ω_d as well as ω_h.

If ω_d has been determined as described in the previous section, the determination of ω_h is easy. Exposure to light of measured intensity is continued until n_d has increased from the steady darkness value n_d to the steady light value n_h determined by the

intensity of the light. Then from the equations for the steady states we have:

$$\frac{\omega_d}{\rho} = \frac{n_d}{1-n_d}, \tag{42}$$

$$\frac{\omega_h}{\rho} = \frac{n_h}{1-n_h}, \tag{44}$$

$$\omega_h = \omega_d \frac{n_h/(1-n_h)}{n_d/(1-n_d)}. \tag{45}$$

It is to be noted that ω_h has been determined for the same cell suspension and for the same external conditions as has ω_d.

In illustration let us take the example from the previous section. There it was found for a cell suspension that

$$\omega_d = 0{\cdot}40\left[\frac{1}{\text{minutes}}\right].$$

The same cell suspension was continuously exposed to light of wave-length 436 mμ, and intensity

$$i = 2{\cdot}72\times10^{-4} \text{ calories/cm.}^2\text{/min.}$$

The n value then increased from

$$n_d = 0{\cdot}25 \quad \text{to} \quad n_h = 0{\cdot}60.$$

According to equation (45) we therefore have

$$\omega_h = 0{\cdot}40\,\frac{0{\cdot}60/0{\cdot}40}{0{\cdot}25/0{\cdot}75} = 1{\cdot}8\left[\frac{1}{\text{minutes}}\right].$$

From this the photochemical decomposition constant for a wave-length of 436 mμ and an intensity $2{\cdot}72\times10^{-4}$ cal./cm.^{2}/min. was

$$z_i = \omega_h - \omega_d = 1{\cdot}8 - 0{\cdot}40 = 1{\cdot}4\left[\frac{1}{\text{minutes}}\right].$$

CHAPTER XII

PHOTOCHEMICAL YIELD

WHEN we came to apply the equation for the photochemical decomposition constant

$$z_i = i\phi\beta, \qquad (5) \text{ Ch. XI}$$

derived from the quantum theory, to the oxygen transporting enzyme, we had to make an assumption for the photochemical yield

$$\phi = \frac{\text{molecules decomposed}}{\text{quanta absorbed}}.$$

This was only possible as a result of model experiments which are described in this chapter. Our most important model substances were the carbon monoxide-haemochromogens from which we measured ϕ and the effect of wave-length on ϕ. Other model substances used were carbon monoxide-ferrocysteine and iron pentacarbonyl. I shall also describe in this chapter quantitative photochemical experiments with chlorophyll which are of importance on account of the close chemical relationship of chlorophyll to the haems.

1. Experiments of Emil Warburg

Emil Warburg was the first to measure photochemical yields. For example, he obtained the following results† for the fission of gaseous hydriodic acid by ultra-violet light:

Wave-length ($m\mu$)	ϕ $\left[\frac{HI}{quanta}\right]$
207	1·0
253	1·05
282	1·05

This means that for each absorbed light quantum, independent of the wave-length, one molecule of hydrogen iodide was decomposed:

$$HI + I\ h\nu = H + I.$$

† E. Warburg, *Berliner Akademieberichte*, 1918, p. 300.

But this result of fundamental importance for the theory of photochemical action was an exception to the general rule. For other photochemical decompositions ϕ was found to be smaller than 1, indeed often much smaller. The photochemical yield was moreover not independent of the wave-length, but generally decreased with increasing wave-length. In gaseous systems ϕ changed with the pressure, and in solution it changed with the nature of the solvent and with the concentration of the dissolved substance. For hydrogen iodide in hexane solution† $\phi = 1$, but in aqueous solution at great dilution $\phi = 0$. For the photochemical fission of potassium nitrate in aqueous solution the following values were obtained:

Wave-length ($m\mu$)	ϕ $\left[\frac{KNO_3}{quanta}\right]$
207	0·25
253	0·17
282	0·024

'Man wird sagen müssen, dass nach den bisherigen Versuchen das Äquivalentgesetz sich für wässrige Lösungen nicht zu bestätigen scheint.'‡

I can offer no reason why what E. Warburg found to be the exception in his apparently simpler experiments was the general rule in our work. We found simple quantum relationships for the most complex organic substances even when they were combined in the living cell. In many cases we found $\phi = 1$; ϕ was always independent of the wave-length.

2. Carbon monoxide-pyridinehaemochromogen

We have determined the photochemical yield ϕ for the decomposition of carbon monoxide-pyridinehaemochromogen,

$$FeCO \rightleftharpoons Fe + CO,$$

using two independent methods; a manometric one in which the light absorption of the haemochromogen solution was complete, and an optical one in which the light absorption was small.

† E. Warburg and W. Rump, *Zeitschr. f. Physik*, **47**, 305 (1928).

‡ E. Warburg, *Photochemie, Handbuch Exper. Physik*, **18**, 619 (1928).

Manometric method†

Into two vessels of the type shown in Fig. 15 is introduced the same amount of a carbon monoxide-haemochromogen solution. The vessels are fitted to a differential manometer, the gas space is filled with a carbon monoxide mixture of known partial pressure, and shaking is continued in a darkened thermostat until equilibrium is reached. One of the two vessels is then exposed to monochromatic light of known intensity, the light entering the bottom of the vessel in a vertical direction. The light is almost completely absorbed by the carbon monoxide-haemochromogen.

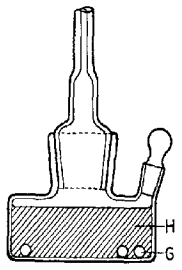


FIG. 15. Manometer vessel.
H = carbon monoxide-haemochromogen solution.
G = glass beads to promote mixing during the rotation of the vessel.

A positive pressure p due to the carbon monoxide formed is developed and increases rapidly to a constant value p_0. At this point the stationary state corresponding to the light intensity employed is reached. It is essential that p_0, the pressure of carbon monoxide produced on exposure, should be small compared with the partial pressure in the original gas mixture.

On removal of the light the pressure of the photochemically produced carbon monoxide is reduced from p_0 to 0. From the rate of this reversal of effect the number of moles CO in the stationary state of exposure which recombine can be calculated. If this rate is ν moles CO per minute and A mole quanta are absorbed per minute in the stationary state, the photochemical yield

$$\phi = \frac{\nu}{A}\left[\frac{\text{moles CO}}{\text{mole quanta}}\right]. \qquad (1)$$

Table 1 contains the results of such determinations calculated from the data in *Bioch. Zeitschr.* **200,** 414 (1928) and **204,** 495 (1929). The wave-length of the light, the intensity, the pressure of the CO, and the temperature were all varied. As follows from

† O. Warburg and E. Negelein, *Bioch. Zeitschr.* **200,** 414 (1928); **204,** 495 (1929).

the quantum theory, all these variations were without influence on ϕ, which was found to lie between 0·72 and 0·82. These figures are so close to 1 that we can assume the deviation from 1 to be due to experimental error and we can write the equation for the decomposition of carbon monoxide-pyridinehaemochromogen as

$$\mathrm{FeCO} + 1\,h\nu = \mathrm{Fe} + \mathrm{CO}. \qquad (2)$$

TABLE 1

Variation	*Temp.* [°C.]	*Total* CO *pressure* [atmos.]	*Wave-length* [mμ]	v [moles CO / minutes]	A [mole quanta / minutes]	$\phi = v/A$ [moles CO / mole quanta]
λ and A	10°	0·045	366	$0{\cdot}774\times10^{-7}$	$1{\cdot}08\times10^{-7}$	0·72
	10°	0·045	436	$1{\cdot}70\times10^{-7}$	$2{\cdot}41\times10^{-7}$	0·71
	10°	0·045	546	$2{\cdot}11\times10^{-7}$	$2{\cdot}84\times10^{-7}$	0·74
CO pressure and A	10°	0·045	546	$2{\cdot}02\times10^{-7}$	$2{\cdot}84\times10^{-7}$	0·71
	10°	0·097	546	$2{\cdot}60\times10^{-7}$	$3{\cdot}22\times10^{-7}$	0·82
	10°	0·195	546	$4{\cdot}54\times10^{-7}$	$5{\cdot}58\times10^{-7}$	0·81
	10°	0·997	546	$29{\cdot}4\times10^{-7}$	$41{\cdot}4\times10^{-7}$	0·71
Temp. and A	4°	0·046	436	$3{\cdot}0\times10^{-7}$	$4{\cdot}06\times10^{-7}$	0·74
	10°	0·046	436	$6{\cdot}15\times10^{-7}$	$8{\cdot}04\times10^{-7}$	0·76

Optical arrangement†

A very dilute solution of carbon monoxide-pyridinehaemochromogen is introduced into a vessel with plane parallel sides. The solution is strongly irradiated in one direction (the narrower cross-section) with the light for the decomposition, and weakly irradiated in the direction at right angles (the greater cross-section) with the measuring light. A wave-length is chosen for the latter that gives as big a difference as possible for the absorption coefficients of the haemochromogen and its carbon monoxide compound. The absorption then changes when carbon monoxide is split off from the haemochromogen, and this change is a measure of the photochemical decomposition (Fig. 16).

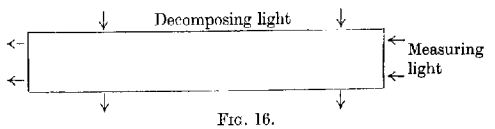


FIG. 16.

With this arrangement Bücher and Negelein have determined

† Th. Bücher and E. Negelein, ibid. **311**, 163 (1942).

the rate at which the light equilibrium is attained in the transition from dark to light, and the dark equilibrium in the transition from light to dark. They found the velocity constants for the decomposition and for the reverse reaction and, using the equation

$$z_i = (\omega_h + \rho) - (\omega_d + \rho), \qquad (15) \text{ Ch. XI}$$

they determined the photochemical decomposition constant of the carbon monoxide-haemochromogen for light intensity i. From the equation

$$z_i = i\phi\beta, \qquad (5) \text{ Ch. XI}$$

in which β is the light absorption coefficient of the carbon monoxide-haemochromogen for the wave-length used (determined spectroscopically in the usual way), they calculated the photochemical yield ϕ.

In the experiments the irradiating light had wave-length 546 mμ. The result was

$$\phi = 1{\cdot}07\left[\frac{\text{moles CO}}{\text{mole quanta}}\right].$$

The quantum equation for the photochemical fission of the carbon monoxide-haemochromogen may therefore be written

$$\text{FeCO} + 1\,h\nu = \text{Fe} + \text{CO}. \qquad (2)$$

The optical method therefore gives the same result as the manometric method. Bearing in mind that the manometric method is purely empirical, but that in the optical method the validity of the mass action laws of Beer and Lambert is assumed, it is obvious that the agreement of the two methods in the value of ϕ is a proof that the theory of the optical method—the theory of the photochemical decomposition constant—is correct in its assumptions and derivation.

3. Carbon monoxide-ferrous cysteine†

The photochemical yield in the decomposition of carbon monoxide-ferrous cysteine,

$$\text{Fe(CO)}_2 \rightleftharpoons \text{Fe} + 2\text{CO},$$

has up till now been determined only by the manometric method. This compound has the advantage over carbon mon-

† O. Warburg and E. Negelein, *Bioch. Zeitschr.* **200**, 414 (1928); **204**, 495 (1929).

oxide-haemochromogen in that the reverse reaction of the fission products proceeds more slowly. Therefore the measurement of the reverse reaction is easier and more accurate. The photochemical yield can, therefore, be more accurately determined.

A further difference between the two substances is the smaller light absorbing power of the cysteine derivative. In the region of the spectrum which we have examined, the absorption of carbon monoxide-ferrous cysteine is one hundred times weaker than carbon monoxide-haemochromogen, so that the photochemical action cannot be determined under conditions of complete absorption of the irradiating light. Actually the light absorption in the vessels (Fig. 17) amounted to only about 5 per cent. of the light used.

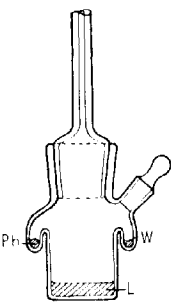


Fig. 17.

Otherwise the arrangement was the same as in the experiments with carbon monoxide-haemochromogen. The solution was exposed to monochromatic light of known intensity till the pressure of the carbon monoxide had become stationary. Then in the dark the rate at which the pressure returned to its previous value was determined. If A mole quanta is absorbed per minute in the stationary state of the exposure, and ν moles CO react per minute in the reverse reaction, then the photochemical yield will be

$$\phi = \frac{\nu}{A}\left[\frac{\text{moles CO}}{\text{mole quanta}}\right].$$

The results for five wave-lengths of the mercury arc calculated from the data in *Bioch. Zeitschr.* **200,** 414 (1928) and **204,** 495 (1929) are collected in Table 2. These results furnish the best proof of the law of photochemical equivalence. For each of the five wave-lengths the photochemical yield ϕ is almost exactly 2 referred to the molecules of carbon monoxide split off, or almost exactly 1 referred to the iron atoms. The quantum equation for the photochemical decomposition of carbon monoxide-ferrous cysteine is therefore

$$\mathrm{Fe(CO)_2} + 1\,h\nu = \mathrm{Fe} + 2\mathrm{CO}. \qquad (3)$$

TABLE 2

Temp. [°C.]	*Total* CO *pressure* [atmos.]	*Wave-length* [mμ]	ν [moles CO / minutes]	A [mole quanta / minutes]	$\phi = \nu/A$ [moles CO / mole quanta]
20	0·488	366	$2{\cdot}44\times10^{-3}$	$1{\cdot}22\times10^{-3}$	2·00
20	0·488	405	$2{\cdot}32\times10^{-3}$	$1{\cdot}21\times10^{-3}$	1·92
20	0·488	436	$2{\cdot}32\times10^{-3}$	$1{\cdot}17\times10^{-3}$	1·98
20	0·488	492	$2{\cdot}50\times10^{-3}$	$1{\cdot}21\times10^{-3}$	2·06
20	0·488	546	$6{\cdot}45\times10^{-3}$	$3{\cdot}50\times10^{-3}$	1·85

4. Iron pentacarbonyl

Our third model substance was iron pentacarbonyl, the light sensitivity of which was discovered in 1905 by Dewar and Jones.† We have measured the photochemical yield in the decomposition of this compound:

$$Fe(CO)_5 = Fe(CO)_4 + 1\,CO$$

for four wave-lengths using the manometric technique.‡ This was particularly easy experimentally since the tetracarbonyl photochemically produced reacts further in the dark reaction

$$Fe(CO)_4 + Fe(CO)_5 = Fe(CO)_9.$$

The carbon monoxide split off cannot therefore react in the reverse direction.

Iron pentacarbonyl which is liquid at room temperature was exposed to light in the undiluted state, that is, in very high concentration, in vessels of the type in Fig. 17. Although the molar coefficient is very small the light absorption was complete on account of the high concentration. The absorbed energy could therefore be taken as equal to the energy of irradiation. On exposure for t minutes to an intensity $J\,\dfrac{\text{mole quanta}}{\text{minutes}}$, the absorbed energy would be Jt mole quanta, and if at the same time x moles carbon monoxide were split off, the photochemical yield would be

$$\phi = \frac{x}{Jt}\left[\frac{\text{moles CO}}{\text{mole quanta}}\right]. \qquad (4)$$

The results are shown in Table 3 and indicate that the photo-

† Dewar and Jones, *Proc. Royal Soc. London*, A **76**, 558 (1905); **79**, 66 (1907).

‡ O. Warburg and E. Negelein, *Bioch. Zeitschr.* **204**, 495 (1929); G. Eyber, *Zeitschr. f. physikalische Chemie*, A **144**, 1 (1929).

chemical yield ϕ is 1 in the ultra-violet and in the blue region. The quantum equation for the photochemical decomposition of iron pentacarbonyl is therefore:

$$Fe(CO)_5 + 1\ h\nu = Fe(CO)_4 + 1\ CO. \qquad (5)$$

TABLE 3

Wave-length [mμ]	x [moles CO]	Jt [mole quanta]	$\phi = x/Jt$ $\left[\frac{\text{moles CO}}{\text{mole quanta}}\right]$
254	$1{\cdot}45 \times 10^{-7}$	$1{\cdot}45 \times 10^{-7}$	1·00
300	$1{\cdot}95 \times 10^{-7}$	$1{\cdot}99 \times 10^{-7}$	0·98
366	$2{\cdot}67 \times 10^{-7}$	$3{\cdot}04 \times 10^{-7}$	0·88
436	$1{\cdot}99 \times 10^{-7}$	$2{\cdot}40 \times 10^{-7}$	0·83

5. Conclusion

The three carbon monoxide-iron compounds, the photochemical dissociation of which has been quantitatively studied, differ from one another in their chemical and optical properties, one could almost say, as much as is possible. For example, the light-absorption coefficients in the blue region are

for carbon monoxide-haemochromogen, of the order of 10^8 $\frac{\text{cm.}^2}{\text{gram atoms Fe}}$;

for carbon monoxide-ferrous cysteine, of the order of 10^6 $\frac{\text{cm.}^2}{\text{gram atoms Fe}}$;

of iron pentacarbonyl, of the order of 10^3 $\frac{\text{cm.}^2}{\text{gram atoms Fe}}$.

The most important external variable in the measurements of the photochemical yield was the wave-length, the shortest being 253 mμ, and the longest 546 mμ. The molar quantum energy was varied therefore from 112,000 to 52,000 calories.

Among other variations that of the concentrations should be emphasized. These were

for the dissociation of carbon monoxide-haemochromogen, about 1/10000 moles/litre;

for the dissociation of carbon monoxide-ferrous cysteine, about 1/1000 moles/litre;

for the dissociation of iron pentacarbonyl, about 4·0 moles/litre.

Since we found with all these variations that one absorbed

light quantum decomposed one molecule of the carbon monoxide-iron compound:

$$FeCO + 1\,h\nu = Fe + CO,$$
$$Fe(CO)_2 + 1\,h\nu = Fe + 2CO,$$
$$Fe(CO)_5 + 1\,h\nu = Fe(CO)_4 + CO,$$

it appears that we can without hesitation take $\phi = 1$ as being the yield in the photochemical dissociation of every carbon monoxide-iron compound. This being so, we had attained our object, which was to show by model experiments which ϕ value should be applicable to the photochemical dissociation of the carbon monoxide compound of the oxygen transporting enzyme.

Nevertheless, in the following chapter we shall deal with one carbon monoxide-iron compound of great biological importance for which the photochemical yield ϕ is not 1.

6. Carbonyl haemoglobin

The photochemical yield in the fission of carbonyl haemoglobin,

$$FeCO \rightleftharpoons Fe + CO, \qquad (6)$$

on account of the great velocity of the reverse reaction, can only be measured if the liberated haemoglobin is removed from the sphere of the reaction. A suitable substance for this purpose is molecular oxygen, which combines reversibly with haemoglobin to give oxyhaemoglobin,

$$Fe + O_2 \rightleftharpoons FeO_2. \qquad (7)$$

If the pressure of oxygen is sufficiently great, an amount of oxyhaemoglobin is formed equal to the amount of carbonyl haemoglobin decomposed. Then the result of combining (6) and (7) is

$$FeCO + O_2 = FeO_2 + CO. \qquad (8)$$

These were the experimental conditions under which Haldane and Smith† discovered the light sensitivity of carbonyl haemoglobin. Carbonyl haemoglobin, of course, in a solution free from oxygen is also decomposed by light, but the decomposition cannot be seen owing to the reverse reaction.

Free haemoglobin formed by the photochemical dissociation

† John Haldane and J. Lorraine Smith, *Journ. Physiology*, **20**, 497 (1896).

of carbonyl haemoglobin can also be removed by reaction with methyl carbylamine. Methyl carbylamine reacts reversibly, as we discovered in 1929,† with haemoglobin according to the equation

$$\text{Fe} + \text{CH}_3\text{NC} \rightleftharpoons \text{Fe}(\text{CH}_3\text{NC}). \qquad (9)$$

If the concentration of the carbylamine is sufficiently great, just as much carbylamine haemoglobin is formed as there was carbonyl haemoglobin decomposed. The resulting reaction follows by combining (6) and (9):

$$\text{FeCO} + \text{CH}_3\text{NC} \rightleftharpoons \text{Fe}(\text{CH}_3\text{NC}) + \text{CO}. \qquad (10)$$

In contrast to Haldane's, this method has the disadvantage that carbylamine is more difficult to obtain, but it has the advantage that carbylamine is a liquid, so that the displacement of the carbon monoxide can be observed and measured manometrically. In the displacement of the carbon monoxide by carbylamine a gas is evolved, but no gas disappears. In the displacement of carbon monoxide by oxygen just as much gas is taken up as is evolved.

We have measured the photochemical yield for the dissociation of carbonyl haemoglobin using both systems; for Haldane's, an optical method, for the second, a manometric method, was used.

Manometric method†

Into two similar vessels of the type shown in Fig. 15 the same amounts of a solution of carbonyl haemoglobin containing 4 per cent. carbylamine are introduced. The vessels are fitted to a differential manometer, both gas spaces are filled with carbon monoxide at atmospheric pressure, and one of the vessels is exposed to light until the pressure of the dissociated carbon monoxide has become constant.

On removing the light the pressure of the dissociated carbon monoxide goes back to zero. The velocity of this reverse reaction gives the amount of carbon monoxide which reacts per

† O. Warburg *et al.*, *Bioch. Zeitschr.* **214**, 26 (1929).

minute in the stationary state of the exposure. If, in this state, ν moles carbon monoxide react per minute and A mole quanta are absorbed per minute by the carbonyl haemoglobin, the photochemical yield

$$\phi = \frac{\nu}{A}\left[\frac{\text{moles CO}}{\text{mole quanta}}\right]. \qquad (1)$$

In all the experiments the wave-length used was that of the yellow mercury line, 578 mμ, the carbon monoxide pressure 0·97 atmospheres, and the pH = 9·5. The light intensity and the concentration of the haemoglobin were varied. At the higher haemoglobin concentrations the light absorption was complete, whilst at the lower concentrations only a small fraction of the light was absorbed. This variation gave a check on the calculation of A, the value for which at great light absorption is quite different from that at low light absorption.

The results of the measurements calculated from the data in *Biochem. Zeitschr.* **214,** 26 (1929) are collected in Table 4. The quantum equation for the photochemical dissociation of carbonyl haemoglobin may therefore be written

$$\text{FeCO} + 4h\nu = \text{Fe} + \text{CO}. \qquad (11)$$

TABLE 4

(578 mμ; pH = 9·5; 10°.)

[Fe] $\left[\frac{\text{gram atoms Fe}}{\text{litre}}\right]$	ν $\left[\frac{\text{moles CO}}{\text{minutes}}\right]$	A $\left[\frac{\text{mole quanta}}{\text{minutes}}\right]$	$\phi = \nu/A$ $\left[\frac{\text{moles CO}}{\text{mole quanta}}\right]$
$0{\cdot}0096 \times 10^{-3}$	$0{\cdot}0294 \times 10^{-6}$	$0{\cdot}116 \times 10^{-6}$	0·25
$1{\cdot}07 \times 10^{-3}$	$0{\cdot}0842 \times 10^{-6}$	$0{\cdot}334 \times 10^{-6}$	0·25
$2{\cdot}40 \times 10^{-3}$	$0{\cdot}856 \times 10^{-6}$	$3{\cdot}54 \times 10^{-6}$	0·24
$1{\cdot}07 \times 10^{-3}$	$0{\cdot}888 \times 10^{-6}$	$3{\cdot}36 \times 10^{-6}$	0·26
$1{\cdot}07 \times 10^{-3}$	$0{\cdot}861 \times 10^{-6}$	$3{\cdot}38 \times 10^{-6}$	0·25

At first this finding, in spite of the regularity and consistency of the results, appeared improbable, and I looked for objections to it (cf. the 1929 publication). But although one might criticize the kinetic theory of the displacement, the method of determining ϕ was a purely empirical one and depended entirely on one consideration, namely, that no greater and no less an

amount of carbonyl haemoglobin can in unit time be dissociated in the stationary state of illumination than that amount of carbon monoxide in the stationary state which recombines in unit time. If this were not so, then the state could not be a stationary one, that is, the pressure of the photochemically dissociated carbon monoxide could not be constant.

Assuming that the determination of ϕ was correct, why did carbonyl haemoglobin behave in a photochemical sense differently from other carbon monoxide-iron compounds, in particular, from the closely related carbon monoxide-pyridine-haemochromogen? Was it the bonding of the haem to the protein, or the fourfold polymerization of the haemoglobin molecule that increased the quantum requirement for the dissociation of one molecule of carbon monoxide from 1 to 4?

In particular, because the quantum number 4 pointed to a possible relationship to that most important photochemical reaction, the decomposition of carbon dioxide in green cells, I again in 1941 took up the photochemical investigation of carbonyl haemoglobin after the problem had lain dormant for twelve years.

Optical method

In 1941 the experimental set-up of 1929 was changed in two ways. The manometric method was replaced by an optical one, and the carbylamine by oxygen. The photochemical yield in the dissociation of carbonyl haemoglobin was therefore determined for the Haldane system†

$$\mathrm{FeCO} + \mathrm{O_2} \rightleftharpoons \mathrm{FeO_2} + \mathrm{CO}. \qquad (8)$$

A very dilute, about 10^{-5} N, solution of haemoglobin was saturated with a mixture of oxygen and carbon monoxide, and illuminated and darkened intermittently. On exposure to light the oxygen replaced the carbon monoxide, and in the dark carbon monoxide replaced the oxygen of the haemoglobin (Fig. 18). The progress of the reaction with time was determined photoelectrically with the apparatus described in section 2 of

† Th. Bücher and E. Negelein, *Bioch. Zeitschr.* **311**, 163 (1942).

this chapter and gave the velocity constants of the displacement reactions

Dark: $$\omega_d+\rho=\frac{1}{t}\ln\frac{n_h-n_d}{n-n_d} \qquad (28)\text{ Ch. XI}$$

Light: $$\omega_h+\rho=\frac{1}{t}\ln\frac{n_d-n_h}{n-n_h} \qquad (28a)\text{ Ch. XI}$$

where the variable n is the fraction of haemoglobin combined

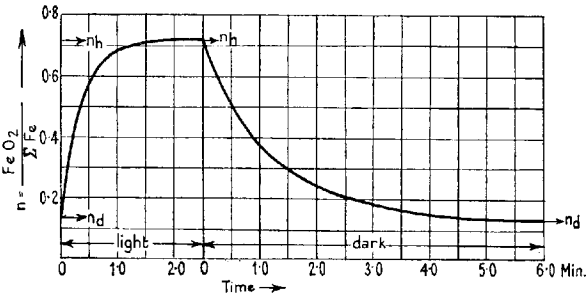


FIG. 18. Effect of light on the partition of haemoglobin.
Light: conversion of carbonyl haemoglobin into oxyhaemoglobin.
Dark: conversion of oxyhaemoglobin into carbonyl haemoglobin.
Abscissa: time.
Ordinate: fraction of total haemoglobin combined with oxygen.

with oxygen at the time t, and the constants n_d and n_h are the n values in the dark and light equilibria.

If $(\omega_d+\rho)$ and $(\omega_h+\rho)$ are calculated from the results which are presented in Table 5, and which are illustrated graphically in Fig. 18, it will be seen that these are constant throughout the whole course of the displacement reactions. This is a proof that the mathematical theory and the technical treatment are in accord.

In illustration of the method the photochemical yield may be calculated from the above figures. If the velocity constant of the dark reaction is subtracted from that of the light reaction we obtain the dissociation constant of the carbonyl haemoglobin for light intensity i and wave-length 546 mμ:

$$z_i=(\omega_h+\rho)-(\omega_d+\rho) \qquad (29)\text{ Ch. XI}$$

$$z_i=2{\cdot}75-0{\cdot}87=1{\cdot}88\left[\frac{1}{\text{minutes}}\right].$$

TABLE 5

Gas mixture: 8 *vol.* % CO *and* 92 *vol.* % O_2. *Temperature*: 4°
Wave-length of light: 546 mμ. pH 8·1

Light			*Dark*		
Light intensity: $J = 1{\cdot}23\times10^{-2}\left[\frac{\text{cal.}}{\text{min. cm.}^2}\right]$ $= 2{\cdot}37\times10^{-7}\left[\frac{\text{mole quanta}}{\text{min. cm.}^2}\right]$					
Time t [min.]	n	$\omega_h+\rho$ [1/min.]	*Time t* [min.]	n	$\omega_d+\rho$ [1/min.]
0	$0{\cdot}133 = n_d$	..	0	$0{\cdot}719 = n_h$	..
0·123	0·299	2·69	0·34	0·568	0·88
0·229	0·407	2·76	0·73	0·442	0·88
0·403	0·525	2·73	1·26	0·330	0·87
0·825	0·659	2·76	2·19	0·227	0·84
∞	$0{\cdot}719 = n_h$	..	∞	$0{\cdot}133 = n_d$	..

Then, on applying the equation

$$z_i = i\phi\beta, \qquad (5) \text{ Ch. XI}$$

we obtain the photochemical yield ϕ if i and β are known.

The light intensity i from the table was

$$2{\cdot}37\times10^{-7}\left[\frac{\text{mole quanta}}{\text{cm.}^2\text{ minutes}}\right].$$

The light-absorption coefficient β of the carbonyl haemoglobin for the wave-length 546 mμ was determined spectroscopically and was $0{\cdot}294\times10^8$. If we substitute for z_i, i, and β in (5) we obtain

$$1{\cdot}88 = 2{\cdot}37\times10^{-7}\,\phi\,2{\cdot}94\times10^7$$

$$\phi = 0{\cdot}27\left[\frac{\text{moles CO}}{\text{mole quanta}}\right].$$

The quantum equation for the dissociation of carbonyl haemoglobin,

$$\text{FeCO} + 4h\nu = \text{Fe} + \text{CO},$$

was therefore proved by a second independent method. All doubts which could have arisen in respect of carbylamine as the replacement substance were set aside, and the problem of the quantum number 4 could be dealt with from a safe experimental foundation.

If the quantum number 4 is associated with the degree of polymerization (4) of the haemoglobin, it ought to be possible

by varying the salt content and the pH to influence the photochemical yield in the dissociation, since K. J. Anderson† of the Svedberg Institute found that, in the presence of salts and at an acid reaction, the fourfold polymerization of the haemoglobin is reduced to twofold polymerization.

The salt concentration was therefore increased from 0·01 to 2·6 molar sodium chloride, and the pH changed from 9·1 to 7·6. Under these conditions Bücher and Negelein obtained the following results on exposure to light of wave-length 546 mμ.

Salt content	pH	$\phi \left[\frac{\text{moles CO}}{\text{mole quanta}}\right]$	$\frac{1}{\phi}\left[\frac{\text{mole quanta}}{\text{moles CO}}\right]$
0·01 molar NaCl	9·1	0·247	4·05
0·01 „ „	9·1	0·230	4·35
0·01 „ „	8·1	0·283	3·53
0·01 „ „	8·1	0·270	3·71
0·01 „ „	7·6	0·296	3·38
1·0 „ „	8·0	0·333	3·00
2·0 „ „	8·1	0·370	2·71
2·6 „ „	8·2	0·376	2·66
2·6 „ „	7·6	0·459	2·18

As can be seen, the quantum requirement changed under the influence of varying salt concentration and pH, and, moreover, in such a way as to reduce the quantum requirement as the degree of polymerization decreased. At the highest salt concentrations and lowest pH which, according to Anderson, would give complete depolymerization to the dimeric haemoglobin a quantum requirement of almost 2 was found.

7. Carbonyl-myoglobin

Myoglobin, the red pigment of muscle, which Hugo Theorell‡ isolated and crystallized in 1932, is closely related chemically to haemoglobin. The prosthetic groups of both pigments are identical, both being protohaem. In both pigments, moreover, the same amount of protein is united with the haem. The iron contents are, therefore, the same. The molecular

† K. J. Anderson in T. Svedberg and K. O. Pedersen, *The Ultracentrifuge*, Oxford University Press, 1940.

‡ Hugo Theorell, *Bioch. Zeitschr.* **252**, 1 (1932); **268**, 46, 55, 64, 73 (1934).

weights are, however, different and in salt-free solution they amount to

Haemoglobin† . . .	$4 \times 17{,}000$
Myoglobin‡ . . .	$1 \times 17{,}000$

Myoglobin therefore, in contrast to haemoglobin, is a non-polymerized haem compound. On account of its simpler structure the gas exchange reactions of myoglobin which Theorell§ and Millikan‖ studied are simpler than the corresponding reactions of haemoglobin.

Myoglobin was of great importance for our problem since it offered us a naturally occurring unpolymerized haemoglobin, and any misgivings which could have arisen on account of the artificial depolymerization by salts would not here apply.

Bücher and Negelein†† examined the photochemical dissociation of carbonyl-myoglobin. They carried out four experiments using the optical method and exposing to light of wave-length 546 mμ. The results were:

Conditions	$\phi \left[\frac{\text{moles CO}}{\text{mole quanta}}\right]$	$\frac{1}{\phi}\left[\frac{\text{mole quanta}}{\text{moles CO}}\right]$
0·02 molar salt; pH 8·2 . .	0·90	1·11
0·02 molar salt; pH 8·2 . .	0·93	1·07
0·02 molar salt; pH 8·2 . .	0·94	1·06
0·02 molar salt; pH 8·2 . .	0·90	1·11

Thus the relationship between the quantum requirement and the degree of polymerization was conclusively proved. Under the conditions of salt concentration and pH which had in this case given a quantum requirement of 1, the quantum requirement for the dissociation of carbonyl haemoglobin had amounted to 3·6.

8. Summary

If we combine all our results on the investigation of the photochemical dissociation of carbonyl-haem compounds, then,

† G. S. Adair, *Journ. biolog. Chem.* **63,** 529 (1925). T. Svedberg and Fåhraeus, *Journ. Amer. Chem. Soc.* **48,** 430 (1926).

‡ T. Svedberg, *Kolloid-Zeitschrift,* **85,** 119 (1938).

§ Hugo Theorell, *Bioch. Zeitschr.* **252,** 1 (1932); **268,** 46, 55, 64, 73 (1934).

‖ G. Millikan, *Proc. Royal Soc.* B **120,** 366 (1936); *Physiological Reviews,* **19,** 503 (1939)'[Baltimore, U.S.A.].

†† Th. Bücher and E. Negelein, *Bioch. Zeitschr.* **311,** 163 (1942).

bearing in mind the degree of polymerization, we obtain the following four quantum equations.

Haemoglobin in salt-free solution . .	$(FeCO)_4 + 4h\nu = Fe_4(CO)_3 + 1\,CO$
Haemoglobin in salt solution . . .	$(FeCO)_2 + 2h\nu = Fe_2(CO)_1 + 1\,CO$
Pyridine-haemochromogen . . .	$(FeCO)_1 + 1h\nu = Fe + 1\,CO$
Myoglobin	$(FeCO)_1 + 1h\nu = Fe + 1\,CO$

9. Chlorophyll

When chlorophyll, exposed to light in acetone solution,† takes up oxygen the photochemical yield $\phi = 1$. Gaffron‡ obtained the following results for four regions of the spectrum:

Wave-length [mμ]	$\phi\left[\frac{\text{moles } O_2}{\text{mole quanta}}\right]$
436	0·99
546	0·97
578	1·0
655	0·97

But when chlorophyll in living plants is exposed to light and reduces carbon dioxide, the photochemical yield $\phi = \frac{1}{4}$, so that the quantum requirement is 4. We found in three regions of the spectrum:§

Wave-length [mμ]	$\phi\left[\frac{\text{moles } CO_2}{\text{mole quanta}}\right]$	$\frac{1}{\phi}\left[\frac{\text{mole quanta}}{\text{moles } CO_2}\right]$
436	0·28	3·6
578	0·23	4·3
660	0·23	4·3

Just as in the case of the dissociation of carbonyl haemoglobin, it is difficult to explain the quantum requirement 4 in this case by the energy requirement, since the following quanta are sufficient for the reduction of 1 molecule of carbon dioxide as a step in carbohydrate synthesis:

in the blue (436 mμ)	1·72 quanta
in the yellow (578 mμ) . . .	2·3 quanta
in the red (660 mμ)	2·6 quanta.

† Dioxan is preferable as the solvent, in manometric experiments at least, when the vapour pressure of the acetone introduces errors.

‡ H. Gaffron, *Chemische Berichte*, **60**, 755, 2229 (1927).

§ O. Warburg and E. Negelein, *Zeitschr. f. physikalische Chemie*, **102**, 236 (1922); **106**, 191 (1923). O. Warburg, *Bioch. Zeitschr.* **166**, 386 (1925). O. Warburg and W. Lüttgens, *Die Naturwissenschaften*, 1944, pp. 161, 301.

From this it is assumed that the energy requirement for carbon dioxide reduction is 112,000 calories. This quantum requirement of 4 for the photochemical reduction of carbon dioxide must depend on a property of the chlorophyll which it possesses in living plant cells but which is absent when it transports oxygen in solution. The question can be asked if this property might possibly be associated with the polymerization of the chlorophyll.

Experiment of W. M. Manning†

W. M. Manning and a staff of co-workers tried to determine the photochemical yield of carbon dioxide by gas analysis. They passed gas mixtures through suspensions of Chlorella which were exposed to light, and analysed the entering and outflowing gas. From very small differences in the composition of the gas mixtures they then calculated the photochemical yield and found a quantum requirement of from 20 to 500 per molecule of carbon dioxide decomposed. They could neither explain this enormous fluctuation, nor could they arrange conditions such as would lessen it. Obviously the gas analysis method is not suitable for experiments of this type, and this view was responsible for my replacing the older method by the manometric one. In our manometric determinations of the quantum requirement the deviation from the mean value in 47 experiments amounted to 10 per cent.

Eichhoff's experiment‡

In 1939 H. J. Eichhoff collaborating with Noddack repeated our manometric measurements of the photochemical carbon dioxide reduction. They used a new method for light absorption measurement which made it possible for them to work with considerably weaker algae suspensions. This was an advantage, because the respiration is of less consequence than the action of the light, and because more dilute cell suspensions are less affected by agitation in the vessels.

† W. M. Manning, J. F. Stauffer, B. M. Duggar, and F. Daniels, *Journ. Amer. Chem. Soc.* **60**, 266 (1938).

‡ H. J. Eichhoff, *Bioch. Zeitschr.* **303**, 112 (1939).

For measuring the photochemical action Eichhoff placed the algae in an alkaline carbonate mixture, which I had suggested in 1919† as being convenient for qualitative assimilation experiments, but which is unsuitable on account of its unphysiological properties for yield determinations. Eichhoff's results varied within wide limits but gave an average value of 4 for the quantum requirement per molecule of carbon dioxide reduced. I distrust this result, however, although it appears to confirm that of our own experiments, because, according to our experience, the quantum requirement of the carbon dioxide reduction in carbonate mixtures is considerably greater than 4.

Robert Emerson's results‡

Emerson and Lewis criticized our manometric measurements of the photochemical reduction of carbon dioxide on the grounds that during the first few minutes in the illumination of the green algae not only is carbon dioxide not taken up but it is actually given off. This photochemical evolution of the carbon dioxide, it was alleged, could be so considerable that three-quarters of our manometric effect might be due not to a reduction but to an evolution of carbon dioxide. Taking this into consideration, 12 quanta and not 4 would be required for the photochemical reduction of one molecule.

In the winter of 1945 I put Emerson's criticisms to test and could not confirm his findings. In the first minutes of the exposure to light no carbon dioxide was evolved. Such an evolution would have been both photochemically and physiologically improbable. The quantum requirement was found to be 4 per molecule of carbon dioxide reduced when the algae were grown in diffused light and when the photochemical yield was measured in a medium, physiological in respect of the algae. (Compare Chapter XXI.)

10. Urease

On exposing solutions of urease to ultra-violet light the enzyme loses its ability to decompose urea. We examined this destruc-

† O. Warburg, *Bioch. Zeitschr.* **100**, 230 (1919).

‡ R. Emerson and Ch. M. Lewis, *Amer. Journ. of Botany*, **26**, 808 (1939).

tion of the enzyme action quantitatively.† Very dilute solutions of crystalline urease containing 0·25 γ of protein per c.c. were exposed to monochromatic ultra-violet light of known intensity. The resulting destruction of the urease was determined by measurement of the enzyme action. If exposure to light of intensity i reduces the activity of the enzyme in time t from W_0 to W the photochemical decomposition constant of the enzyme would be

$$z_i = \frac{1}{t}\ln\frac{W_0}{W}. \qquad (6)\ \text{Ch. XI}$$

For calculation of the photochemical yield using the equation

$$z_i = i\phi\beta \qquad (5)\ \text{Ch. XI}$$

a value for the molecular weight of urease, which was needed to find the light absorption coefficient β, was not available in 1933. The molecular weight of urease has now, however, been determined by Sumner‡ and is 480,000, so that equation (5) can be used and the photochemical yield ϕ for the destruction of urease can be calculated. The results are set out in Table 6.

TABLE 6

Wave-length [mμ]	$\beta\left[\frac{\text{cm.}^2}{\text{moles urease}}\right]$	$\frac{1}{\phi}\left[\frac{\text{mole quanta absorbed}}{\text{moles urease destroyed}}\right]$
196	850×10^8	302
207	268×10^8	326
228	63×10^8	280
254	$4{\cdot}0\times10^8$	150
283	$4{\cdot}8\times10^8$	264
366	$0{\cdot}18\times10^8$	260

From the table it can be seen that on an average 300 quanta must be absorbed to make a molecule of urease inactive. This result is remarkable from several points of view. If, for example, we compare the longest and the shortest wave-lengths we find that the light absorption at 196 mμ is 4,700 times greater than at 366 mμ. At 366 mμ only tryptophan and tyrosine really absorb light, whilst at 196 mμ all the amino acids of the enzyme

† F. Kubowitz and E. Haas, *Bioch. Zeitschr.* **257**, 337 (1933).
‡ J. B. Sumner *et al.*, *Journ. biolog. Chem.* **125**, 37 (1938).

molecule are absorbing. At 366 mμ the molar quantum energy amounts to 77,500 calories, whilst at 196 mμ the value is 145,000 calories. In spite of this, the quantum requirement in both regions of the spectrum is almost the same, so that it follows that for the destructive action of the light it is immaterial which part of the enzyme molecule is absorbing and how great is the energy of the quanta.

We shall continue this experiment, but not with urease, because it is not known why urease has its particular action. We shall use an enzyme the active group of which is known. In 1933 when we examined the photochemical destruction of urease such enzymes had not been isolated.

CHAPTER XIII

ABSORPTION SPECTRUM OF THE OXYGEN TRANSPORTING ENZYME

In our study of the photochemistry of carbon monoxide-iron compounds we shall now deal with the oxygen transporting enzyme, that is, not with an isolated enzyme, but with a biologically active one. Difficulties will arise because what could

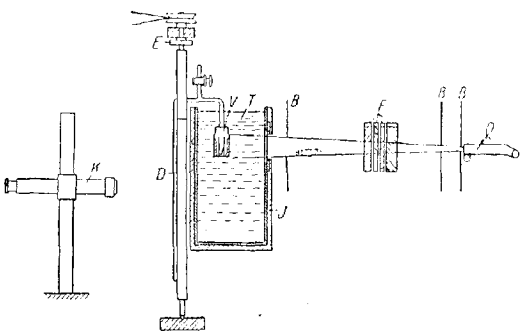


FIG. 19. Determination of the light action.

K = cathetometer. D = differential manometer.
E = eccentric pulley. V = quartz vessel.
T = thermostat made from lead plate with a quartz window.
J = felt jacket. Q = mercury vapour lamp.
B = shutters. F = light filter.

be directly measured in model experiments will now have to be calculated and assessed by observation of the effect on biological processes. Inhibition of cell respiration by carbon monoxide, the action of light on such inhibition, and the effect of the intensity and wave-length of the light, are the experimental factors from which the chemical nature and the mechanism of action of the oxygen transporting enzyme will be deduced.

The experimental arrangement is shown in Figs. 19 and 20. Torula yeast and acetic acid bacteria were used as experimental

material because these cells have a large oxygen respiration, and they stand up to the vigorous shaking necessary in manometric experiments for a sufficiently long time without ill effects.

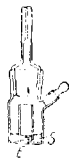


FIG. 20. Quartz vessel
S = 2 c.c. cell suspension.
E = inner vessel containing 0·2 c.c. 5% potash.

1. Light sensitivity

When cells, the respiration of which has been inhibited by carbon monoxide, are exposed to light the partition constant K increases from the value in the dark to a light value determined by the intensity and the wave-length,

$$\text{dark:} \quad K_d = \frac{n_d}{1-n_d}\frac{\text{CO}}{\text{O}_2} \tag{1}$$

$$\text{light:} \quad K_h = \frac{n_h}{1-n_h}\frac{\text{CO}}{\text{O}_2}. \tag{2}$$

If the pressure of oxygen and carbon monoxide remain constant the difference (2) minus (1) is

$$K_h - K_d = \Delta K = \frac{\text{CO}}{\text{O}_2}\left(\frac{n_h}{1-n_h} - \frac{n_d}{1-n_d}\right), \tag{3}$$

and if we divide (3) by (1) to eliminate $\frac{\text{CO}}{\text{O}_2}$ we obtain

$$\frac{\Delta K}{K_d} = \frac{n_h/(1-n_h) - n_d/(1-n_d)}{n_d/(1-n_d)}. \tag{4}$$

If one therefore determines for cells, the respiration of which has been inhibited by carbon monoxide, the residual respiration in the dark n_d and that in light n_h, the percentage displacement of the partition constant $\frac{\Delta K}{K_d}$ can be calculated from (4).

The quantity $\frac{\Delta K}{K_d}$ has the important significance, as is theoretically established below, that it is proportional to the light intensity for any given cell suspension and wave-length. Therefore

$$\frac{\Delta K}{K_d} = Li, \tag{5}$$

where L is a proportionality factor which we call 'light sensitivity', the dimensions of which are a reciprocal of intensity.

In Fig. 21 an experimental example is illustrated graphically.† $\frac{\Delta K}{K_d}$ plotted as a function of the light intensity gives nearly a straight line which goes through the origin. The proportionality factor in equation (5), the light sensitivity L, has an interesting physical significance. On substituting for the partition constant

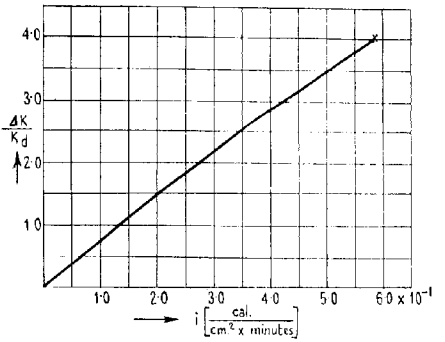


FIG. 21. Displacement of the partition constant K by light of wave-length 436 mμ. Temp. 10°.

K the four velocity constants by which it is determined—compare Chapter XI, equation (32),

$$K = \frac{B}{Z'}\frac{z}{b} \tag{6}$$

—it has to be remembered that on exposure to light only one of the four velocity constants changes, viz. z, the velocity constant of the FeCO dissociation. Therefore, resolving (6) for darkness and for light we obtain

$$K_d = \frac{B}{Z'}\frac{z_d}{b},$$

$$K_h = \frac{B}{Z'}\frac{z_h}{b}.$$

Hence

$$K_h - K_d = \Delta K = \frac{B}{Z'}\frac{1}{b}(z_h - z_d),$$

$$\frac{\Delta K}{K_d} = \frac{z_h - z_d}{z_d}. \tag{7}$$

† O. Warburg and E. Negelein, *Bioch. Zeitschr.* **193**, 339 (1928).

But since $(z_h - z_d)$ is the photochemical decomposition constant of the carbon monoxide compound of the enzyme we can write instead of (7)

$$\frac{\Delta K}{K_d} = \frac{z_i}{z_d}. \tag{8}$$

If we eliminate z_i from this, using equation (5) of Chapter XI,

$$z_i = i\phi\beta,$$

we obtain

$$\frac{\Delta K}{K_d} = i\frac{\phi\beta}{z_d}. \tag{9}$$

We also have a value for $\frac{\Delta K}{K_d}$ in equation (5)

$$\frac{\Delta K}{K_d} = iL. \tag{5}$$

Therefore, from (9) and (5), we obtain the important equation†

$$L = \frac{\phi\beta}{z_d}\left[\frac{\text{cm.}^2 \text{ minutes}}{\text{mole quanta}}\right]. \tag{10}$$

The light sensitivity therefore increases with increase of β, the light absorption coefficient of the carbon monoxide compound of the enzyme, and with increase of ϕ, the photochemical yield in the dissociation of the carbon monoxide compound of the enzyme. The light sensitivity decreases with increase of z_d, the darkness dissociation constant of the carbon monoxide compound, since the more rapid the darkness dissociation is, the less will any additional photochemical decomposition change the darkness partition of the enzyme. On the other hand, the light sensitivity is independent of the pressure of oxygen and carbon monoxide and also independent of the velocity constants Z', B, and b.

It is a consequence of equation (10) that the light sensitivity should change with temperature, and moreover it will increase if the temperature decreases, because if the temperature decreases z_d becomes smaller, whilst ϕ and β remain constant. Therefore in experiments depending on high light sensitivity the temperature should be kept as low as possible, a factor which

† O. Warburg *et al.*, *Bioch. Zeitschr.* **214**, 26 (1929); **214**, 64 (1929).

we have taken into account in the determination of the absorption coefficient of the enzyme.

Since the light sensitivity L is determined by the three theoretically important quantities ϕ, β, and z_d

$$L = \frac{\phi\beta}{z_d}, \tag{10}$$

and since L can always be easily determined from the stationary partition values n_d and n_h, and the light sensitivity i is always easy to determine,

$$L = \frac{1}{i}\frac{\Delta K}{K_d}. \tag{5}$$

L has therefore played a great part in the photochemical investigation of the oxygen transporting enzyme. For example, we found the following values from equation (5) when the light had a quantum intensity i and n increased from n_d to n_h:

Carbon monoxide compound of substance	*Temp.* [°C.]	*Wave-length* [mμ]	L $\left[\frac{\text{cm.}^2 \text{ minutes}}{\text{mole quanta}}\right]$
Oxygen transporting enzyme of acetic acid bacteria . .	10	436	$2{\cdot}1 \times 10^8$
Oxygen transporting enzyme of yeast	0	436	34×10^8
Oxygen transporting enzyme of yeast	10	436	$7{\cdot}6 \times 10^8$
Oxygen transporting enzyme of yeast	10	546	$0{\cdot}73 \times 10^8$
Haemoglobin	4	546	$0{\cdot}67 \times 10^8$
Haemoglobin in N carbylamine .	10	578	$2{\cdot}0 \times 10^8$

In regard to these figures it is to be noted that the light sensitivity is not only different for different kinds of cells but varies for the same cells when these have been cultured in different ways. When, therefore, it is desired to examine the effect of wave-length or temperature on the light sensitivity it is best to use aliquots of the same cell suspension.

We found that the light sensitivity L of the carbon monoxide compound of the yeast enzyme was of the order of 10^9 (expressed as reciprocal quanta intensity) for the wave-length 436 mμ and a temperature of 10°. A value for L of 10^9 means that the partition

constant K is 10^9 times its dark value when the cells are exposed to light of quantum intensity $i = 1$.

For measurements of the action spectrum it is, however, sufficient to obtain a twofold increase in the partition constant. Then

$$\Delta K = K_d$$

and the general equation

$$L = \frac{1}{i}\frac{\Delta K}{K_d} \tag{5}$$

becomes $L = \frac{1}{i_*}$ $\quad i_* = i$ when $\Delta K = K_d$.

$\frac{1}{L}$ is therefore the quantum intensity which doubles the partition constant, and for yeast, using the wave-length 436 mμ and at temperature 10°, it has the value

$$i_* = \frac{1}{L} = \frac{1}{10^9} = 10^{-9}\left[\frac{\text{mole quanta}}{\text{cm.}^2\ \text{minutes}}\right]$$
$$= 0{\cdot}65 \times 10^{-4}\left[\frac{\text{cal.}}{\text{cm.}^2\ \text{minutes}}\right].$$

This is about 1/10,000 of the intensity of the sun on the surface of the earth.

2. Relative absorption spectrum†

When cells, the respiration of which has been inhibited by carbon monoxide, are exposed to light of wave-lengths λ_1 and λ_2 and the light sensitivities are found to be L_1 and L_2, on applying equation (10) we obtain

$$L_1 = \frac{\phi_1 \beta_1}{z_d}, \tag{10a}$$

$$L_2 = \frac{\phi_2 \beta_2}{z_d}, \tag{10b}$$

where the dark dissociation constant z_d has no index, since for a given cell suspension z_d must be the same in both cases. The photochemical yield ϕ_1 could be different from ϕ_2. Since, however, in the photochemical dissociation of carbon monoxide

† O. Warburg and E. Negelein, *Bioch. Zeitschr.* **193**, 339 (1928); **214**, 64 (1929). O. Warburg, *Zeitschr. für angewandte Chemie*, **45**, 1 (1932). F. Kubowitz and E. Haas, *Bioch. Zeitschr.* **255**, 247 (1932).

iron compounds we have never found any change of ϕ with wave-lengths in the region of the spectrum from 253 to 578 mμ, then

$$\phi_1 = \phi_2.$$

Dividing (10a) by (10b) we obtain

$$\frac{L_1}{L_2} = \frac{\beta_1}{\beta_2}. \tag{11}$$

This is the equation for the calculation of the relative absorption spectrum of the carbon monoxide compound of the oxygen transporting enzyme.

The manometric technique was so arranged that the ratio of the light sensitivities L, that is, the ratio of the absorption coefficients β, could be determined with an accuracy of 10 per cent. The optical part of the work was more difficult. It was not easy to isolate a sufficient number of regions of the spectrum of satisfactory purity and intensity. We began our experiment with six regions and finished with thirty. The mercury lamp, spark discharges, and carbon arcs were used as the light sources, from which the particular regions of the spectrum were isolated with the help of filters and monochromators. Our shortest wave-length, 253 mμ, was obtained using a zinc spark, and our longest, 671 mμ, was obtained from a lithium-carbon arc. Probably no other photochemical reaction has been investigated using so many wave-lengths.

The results of one series of experiments using yeast cells are illustrated in Fig. 22. Leaving out of consideration for the present the dimensions of the ordinates, let us deal with the relative spectrum. This spectrum—the absorption spectrum of the carbon monoxide compound of the oxygen transporting enzyme—is very similar to that for carbonyl haemoglobin shown in Fig. 23. It differs from the latter, however, in that the bands have been displaced towards the red end, and in the height of the ϵ-bands. The position of the bands is approximately as follows:

	ϵ	δ	γ	β'	β	α
Carbon monoxide enzyme	283	356	430	512	540	590
Carbonyl haemoglobin	275	344	420	..	540	570

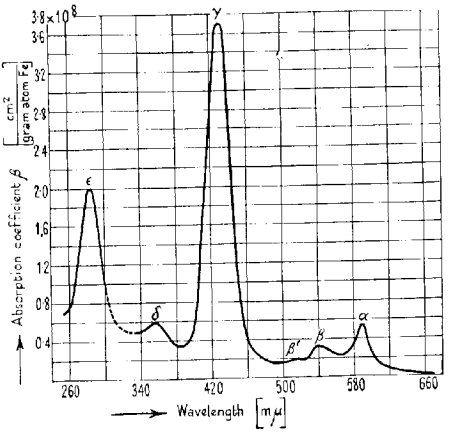


FIG. 22. Carbon monoxide compound of the oxygen transporting respiratory enzyme.

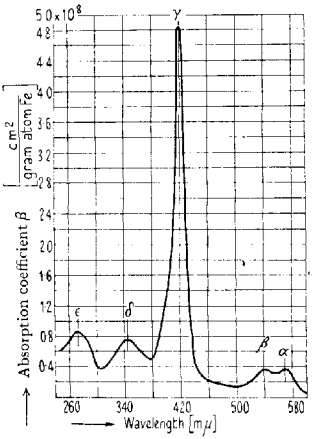


FIG. 23. Carbonyl haemoglobin.

We obtained the same absorption spectrum for the oxygen transporting enzyme of acetic acid bacteria. As an example of animal cells we investigated the retina.† The oxygen transporting enzyme of this material had a spectrum displaced towards the red in comparison with that of haemoglobin.

3. Molar absorption spectrum

When cells, the respiration of which has been inhibited by carbon monoxide, are exposed to monochromatic light of known intensity the light sensitivity L_λ for the wave-length λ is obtained. When the cells are darkened, and the time taken to reach the dark equilibrium is determined, the dark dissociation constant z_d of the carbon monoxide compound of the enzyme is obtained. Equation (10)

$$L_\lambda = \frac{\phi\beta_\lambda}{z_d} \tag{10}$$

gives the product $\phi\beta_\lambda$, and if we put $\phi = 1$ we obtain from (10) since

$$\beta_\lambda = \frac{L_\lambda z_d}{1}$$

the molar absorption coefficient β_λ of the carbon monoxide compound of the oxygen transporting enzyme.

In four experiments with four different yeast suspensions, using the blue mercury line 436 mμ at 10° and with $\phi = 1$, we found:‡

$L_{436}\left[\frac{\text{cm.}^2\text{ min.}}{\text{mole quanta}}\right]$	$z_d\left[\frac{1}{\text{min.}}\right]$	$\beta_{436} = \frac{L_{436}\, z_d}{\phi}\left[\frac{\text{cm.}^2}{\text{gram atoms Fe}}\right]$
$7{\cdot}9\times10^8$	0·526	$4{\cdot}18\times10^8$
$7{\cdot}4\times10^8$	0·382	$2{\cdot}82\times10^8$
$6{\cdot}9\times10^8$	0·575	$3{\cdot}96\times10^8$
$8{\cdot}3\times10^8$	0·402	$3{\cdot}32\times10^8$
		Mean $3{\cdot}6\times10^8$

The mean value of β_{436} was therefore $3{\cdot}6\times10^8$. Using this value to convert the relative into the molar absorption spectrum we obtained the molar absorption spectrum of the carbon monoxide compound of the oxygen transporting enzyme shown in Fig. 22.

† O. Warburg and E. Negelein, *Biochem. Zeitschr.* **214**, 101 (1929).
‡ Id., ibid. 64 (1929).

If we compare this with the molar absorption spectrum of carbonyl haemoglobin shown in Fig. 23 it can be seen that the two spectra are almost identical not only in their composition but also in the heights of the bands. Only in the heights of the ϵ-bands which are due partly to the absorption by the protein do they show any real difference. This will be discussed in the next section.

If in the calculation of the molar spectrum of the enzyme we had taken $\phi = 0{\cdot}50$ or $\phi = 0{\cdot}25$, then since

$$\beta = \frac{Lz_d}{\phi}$$

the enzyme bands would have become twice or four times as high as the corresponding haemoglobin bands. That is to say, the molar enzyme spectrum would have been too high to be a haemochromogen spectrum. Although, therefore, on the basis of the model experiments† of recent years (which differ from those of 1929) values for $\phi = 0{\cdot}50$ and $0{\cdot}25$ must also be taken into consideration, $\phi = 1$ is retained because only with this value can the relative and the molar enzyme spectra be brought into agreement.

I almost regret that we cannot put $\phi = 0{\cdot}25$ and thus assume that the enzyme is a fourfold polymerized haem compound. On such a basis the oxidation of the enzyme iron, by which four atoms of ferrous iron react with one molecule of oxygen, could be better understood, and also a reason could be furnished as to why haemoglobin, a successor to the oxygen transporting enzyme, is a fourfold polymer.

Note. In the first determination of the molar absorption coefficients of the oxygen transporting enzyme the light intensities were wrongly determined‡ due to a defect in the wiring of the bolometer. In spite of this, however, we found the correct value for these coefficients. I have been asked why we measured the light intensities in the determination of the molar absorption coefficients since they cancelled out in the result.

The answer is that we made two independent measurements with the faulty bolometer. We determined the photochemical

† Cf. Chapter XII.

‡ O. Warburg and E. Negelein, *Bioch. Zeitschr.* **204**, 495 (1929).

yield ϕ for the model substances and the light sensitivity L for the enzyme. Both values were 2·2 times too large. Since in the calculation of the molar absorption coefficients β,

$$\beta = \frac{Lz_d}{\phi}, \tag{10}$$

the ratio L/ϕ comes in, the error cancelled itself out, but the intensities did not.

4. Protein component

If the ϵ-bands in Figs. 22 and 23 are compared it is seen that the enzyme band is considerably higher than that of haemoglobin. Since the bands are due partly to the haem and partly to the protein, these differing heights mean that more protein is combined with the haem in the enzyme than in haemoglobin. How much greater this amount is can be calculated as follows:

In the region of the ϵ-bands the 'molar' absorption coefficient of the carbonyl haemoglobin is $0{\cdot}83\times10^8\left[\frac{\text{cm.}^2}{\text{mole Fe}}\right]$, and that of the carbon monoxide haem $0{\cdot}5\times10^8\left[\frac{\text{cm.}^2}{\text{mole Fe}}\right]$. The molar absorption coefficient of the protein component of haemoglobin is therefore

$$(\beta)_{\text{molar}}^{\text{protein}} = 0{\cdot}83\times10^8 - 0{\cdot}50\times10^8 = 0{\cdot}33\times10^8\left[\frac{\text{cm.}^2}{\text{mole Fe}}\right].$$

On the other hand, in our experience the absorption coefficient referred to unit weight of most enzyme proteins in the region of the ϵ-band is

$$(\beta)_{\text{g.}}^{\text{protein}} = 2{\cdot}0\times10^3\left[\frac{\text{cm.}^2}{\text{g. protein}}\right].$$

If we divide the molar β by the β referred to unit weight, we obtain the amount of protein combined with one gram atom of iron in haemoglobin:

$$\frac{(\beta)_{\text{molar}}^{\text{protein}}}{(\beta)_{\text{g.}}^{\text{protein}}} = \frac{0{\cdot}33\times10^8}{2{\cdot}0\times10^3} = 16500\left[\frac{\text{g. protein}}{\text{gram atoms Fe}}\right].$$

Applying the same method of calculation to the ϵ-band of the enzyme we obtain

$$(\beta)^{\text{protein}}_{\text{molar}} = 2{\cdot}0\times10^{8} - 0{\cdot}5\times10^{8}$$

$$= 1{\cdot}5\times10^{8}\left[\frac{\text{cm.}^2}{\text{gram atoms iron}}\right];$$

and the amount of protein which is combined with 1 gram atom iron in the enzyme is

$$\frac{(\beta)^{\text{protein}}_{\text{molar}}}{(\beta)^{\text{protein}}_{\text{g.}}} = \frac{1{\cdot}5\times10^{8}}{2{\cdot}0\times10^{3}} = 75000\left[\frac{\text{g. protein}}{\text{gram atoms Fe}}\right].$$

The objection to this method of calculation is that $(\beta)^{\text{protein}}_{\text{g.}}$ (the value of which depends on the content of the protein in tryptophane and tyrosine) has not been measured for the enzyme protein and need not necessarily be 2×10^{3}. However, it is probable that the value lies near 2×10^{3} and that our calculation gives the correct order of magnitude.

5. Concentration of the enzyme iron

Since the carbon monoxide inhibition of cell respiration is independent of the concentration of the enzyme iron, it is impossible by inhibitory experiments to find how much oxygen transporting iron is contained in the cells. The enzyme concentration does not appear in any of our equations. It is only the values of the ratios

$$\frac{\text{ferric}}{\text{ferrous}} \text{ or } \frac{\text{ferric}}{\text{FeCO}} \text{ or } \frac{\text{ferric}}{\sum\text{Fe}}$$

which can be determined by our respiration measurements.

On the other hand, when aerobic cells are brought into contact with carbon monoxide they must combine with an amount of this substance equivalent to that of the oxygen transporting enzyme

$$\text{Fe} + \text{CO} = \text{FeCO}.$$

If one could determine this carbon monoxide, one could obtain a maximum value for the concentration of enzyme iron in the cells.

5 g. of yeast suspended in phosphate were added to vessels I and II of the differential manometer† shown in Fig. 24. The gas space in vessel I was filled with nitrogen, that of vessel II with nitrogen containing 5 per cent. carbon monoxide. When the vessels had been shaken till the differential manometer showed no further pressure change, the same amount of carbon monoxide

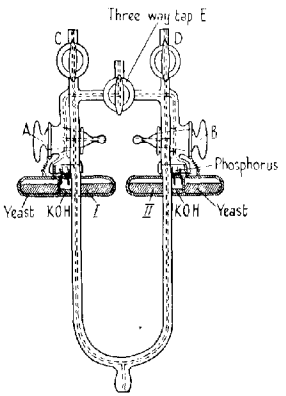


FIG. 24. Differential manometer for the determination of very small gas absorptions.

was introduced under pressure into both vessels, and shaking was continued. Vessels I and II must contain the same amount of dissolved carbon monoxide, since the increase of carbon monoxide pressure was the same in both. In addition to this, however, some of the carbon monoxide introduced would be chemically bound in vessel I due to the reaction

$$Fe + CO = FeCO,$$

whilst in vessel II this reaction would already have taken place before the introduction of the carbon monoxide.

The differential manometer showed no difference in levels in any experiment. Taking into consideration the accuracy of measurement and the constants for the vessels, this meant that

† O. Warburg and F. Kubowitz, *Bioch. Zeitschr.* **203**, 95 (1928).

5 g. of yeast cells reacted chemically with less than $0{\cdot}16\times10^{-3}$ c.c. of carbon monoxide. The concentration of enzyme iron in the yeast cells cannot therefore be greater than

$$[\mathrm{Fe}] = 1{\cdot}43\times10^{-9}\left[\frac{\text{gram atoms iron}}{\text{cm.}^3}\right].$$

The concentration of cytochrome in yeast cells is more than ten times greater than this value, so that the differential manometer would have shown a difference in levels if the carbon monoxide had reacted with the cytochrome. Our spectroscopic observations made in 1926 which showed that cytochrome does not react with carbon monoxide were therefore confirmed by an independent method.

6. Velocity of reaction of the enzyme iron†

When the oxygen transporting enzyme transports oxygen in respiring cells, the respiration, which is a stationary condition, equals the velocity with which the ferrous iron is oxidized and also that with which the ferric iron is reduced. If we designate the respiration as A and the oxygen pressure as p_{O_2} then

$$A = 0{\cdot}25\times B\times[\text{ferrous}]\times p_{O_2}, \tag{12}$$

$$A = 0{\cdot}25\times Z'\times[\text{ferric}], \tag{13}$$

where B and Z' are the velocity constants of the oxidation and reduction of the enzyme iron and the factor 0·25 has the dimension $\left[\frac{\text{moles } O_2}{\text{moles iron}}\right]$. From these equations the velocity constants B and Z' can be calculated if the concentration of the ferrous and ferric iron and the oxygen pressure are known. For baker's yeast at 10° in air, in which the whole of the enzyme iron is present as ferric iron, we found

$$A = 0{\cdot}45\times10^{-5}\left[\frac{\text{moles } O_2}{\text{minutes.cm.}^3\text{ yeast}}\right].$$

Substituting the maximum value for enzyme iron found above,

$$[\textstyle\sum \mathrm{Fe}] = 1{\cdot}43\times10^{-9}\left[\frac{\text{moles Fe}}{\text{cm.}^3\text{ yeast}}\right],$$

† O. Warburg and F. Kubowitz, *Bioch. Zeitschr.* **202**, 387 (1928); **203**, 95 (1928); **214**, 5 (1929).

equation (13) becomes

$$Z' = \frac{0{\cdot}45 \times 10^{-5}}{1{\cdot}43 \times 10^{-5} \times 0{\cdot}25} = 1{\cdot}3 \times 10^{4} \left[\frac{1}{\text{minutes}}\right].$$

For baker's yeast at 10° under an oxygen pressure at which the respiration was half as great as it was in air, that is, under conditions such that [ferrous] = [ferric], we found

$$p'_{O_2} = 3 \times 10^{-4} \text{ [atmospheres]}.$$

Substituting this value in (12), from (12) and (13) we obtain

$$B = \frac{Z'}{p'_{O_2}} = \frac{1{\cdot}3 \times 10^{4}}{3 \times 10^{-4}} = 0{\cdot}43 \times 10^{8} \left[\frac{1}{\text{minutes atmospheres}}\right].$$

The values of B and Z' thus obtained are minimum values, since they were obtained using the maximum value for the enzyme concentration [$\sum$ Fe].

CHAPTER XIV

SPECTROSCOPIC IDENTIFICATION OF THE OXYGEN TRANSPORTING ENZYME

EVER since it was known that carbon monoxide inhibited cell respiration we have been examining cells spectroscopically with a view to finding bands which were displaced by carbon monoxide, but it was not until 1933, eight years after the discovery of carbon monoxide inhibition, that we found such bands.

The choice of experimental material was probably the determining factor. On the assumption that the stronger the respiration the greater would be the amount of oxygen transporting enzyme in the cell, the most promising materials were the very strongly respiring cells. Acetic acid bacteria and, according to the measurements of Meyerhof and Burke,† azotobacter belonged to this class.

1. Acetic acid bacteria‡

The vertical spiral of a low-voltage metal filament lamp is set up opposite the centre of a quartz vessel, the plane parallel sides of which are 1 cm. apart. The vessel contains the acetic acid bacteria suspension. The slit of a hand spectroscope is placed as near as possible to the side of the vessel away from the lamp (Fig. 25).

When the cell suspension is 8 per cent. by volume, three cytochrome bands in the green region at 550, 553, and 563 mμ are seen under anaerobic conditions. A cytochrome band at 604 mμ which can be seen in most other cells is missing.

The cytochrome bands of the acetic acid bacteria have the usual properties. They disappear on going over to aerobic conditions. They are not displaced by carbon monoxide, not even by carbon monoxide at 60 atmos. pressure.§ In addition, they are not displaced by cyanide. Both carbon monoxide and

† O. Meyerhof and D. Burke, *Zeitschr. f. physikalische Chemie*, A **139**, 117 (1928); O. Meyerhof and W. Schulz, *Bioch. Zeitschr.* **250**, 35 (1932).

‡ O. Warburg and E. Negelein, ibid. **262**, 237 (1933); O. Warburg, E. Negelein, and E. Haas, ibid. **266**, 1 (1933).

§ A. Reid, ibid. **242**, 159 (1931).

cyanide, by blocking the oxygen transporting enzyme, prevent the disappearance of the cytochrome bands on changing over to aerobic conditions.

When the concentration of the acetic acid bacteria is raised from 8 per cent. to 25 per cent. by volume, there appears under anaerobic conditions, in addition to the now very strong cytochrome bands, a weak band in the yellow at 589 mμ. This band is displaced by carbon monoxide to 593 mμ, that is, to the

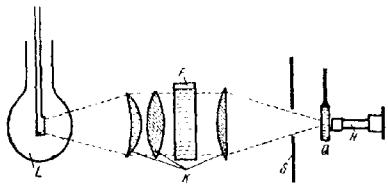


FIG. 25. L = half-watt lamp with vertical filament (15 volt, 11 amp.); K = condenser; F = heat filter (20% acid solution of ferrous sulphate, 3 cm.); Q = quartz vessel with capillary stopper containing the cell suspension; H = hand spectroscope.

region of the spectrum in which, according to our photochemical measurements, the α-band of the carbon monoxide compound of the oxygen transporting enzyme is situated.

In other ways, too, the band in the yellow behaves just as a band of the oxygen transporting enzyme should. In particular, it disappears on changing over to aerobic conditions in the presence of cyanide. It behaves, therefore, towards cyanide differently from the cytochrome bands.

The origin of a band in the red at 639 mμ which we have discovered under aerobic conditions at respiration-inhibiting concentrations of cyanide is still not clear. Lemberg and Wyndham† have expressed the view that this band originates from a green haemin.‡ Against such a theory there is the evidence that the band in the red disappears again on washing out the cyanide from the bacteria, whereas we have no information to the effect that the oxidation reaction by which the green haemins are formed is reversible.

† R. Lemberg and R. A. Wyndham, *Proc. Royal Soc. New South Wales*, **70**, 343 (1937). ‡ O. Warburg and E. Negelein, *Chem. Berichte*, **63**, 1816 (1930).

2. Azotobacter†

When azotobacter is examined spectroscopically using the arrangement in Fig. 25, two cytochrome bands at 550 and 563 mμ are observed under anaerobic conditions. These bands behave towards oxygen, carbon monoxide, and cyanide in the same way as the cytochrome bands of the acetic acid bacteria.

In addition to those of cytochrome, however, a sharp band in

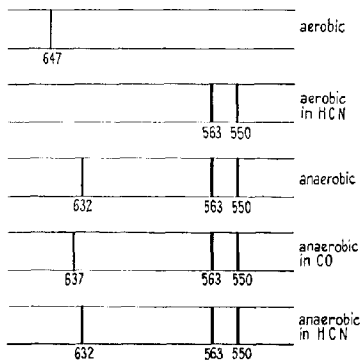


FIG. 26. The respiration of azotobacter and its inhibition by CO and HCN. Diagram of spectra.

the red at 632 mμ can be seen; this band has the interesting property of being displaced by carbon monoxide to the extent of 5 mμ, i.e. to 637 mμ. The band at 637 mμ is also a sharp band and in other ways too it behaves like the band of the oxygen transporting enzyme. In changing over to aerobic conditions it disappears and a band at 647 mμ is seen. This corresponds to the change from ferrous to ferric iron. Cyanide has no effect on the 632 band but causes the 647 band to disappear. Cyanide does not react with ferrous, but only with ferric iron, as is shown by the effect on haemoglobin and methaemoglobin and the formation of cyanhaemoglobin.

These results are illustrated graphically in Fig. 26. The

† E. Negelein and W. Gerischer, *Naturwissenschaften*, **21**, 884 (1933); *Bioch. Zeitschr.* **268**, 1 (1934).

experiments should be carried out by all those who are studying the respiration problem, for here one can see with the spectroscope how the oxygen transporting enzyme transports oxygen by virtue of the valency change ferrous → ferric, and how carbon monoxide by reacting with the ferrous iron, and cyanide by reacting with the ferric iron, inhibit the oxygen respiration.

3. Heart muscle

In 1939 Keilin and Hartree† were able to identify in heart muscle preparations the band of the carbon monoxide compound of the enzyme at 590 mμ which we had found spectroscopically using the acetic acid bacteria in 1933.‡ They made an interesting observation when, according to our recommendation,§ they used blue light. They saw how, on passing carbon monoxide, the main part of band 448 mμ changed over to 431 mμ, i.e. to the region in which the main band of the carbon monoxide compound of the enzyme is situated. From that it can be concluded that the greater part of the band at 448 mμ is the main band of the ferrous form of the oxygen transporting enzyme.

The work of Keilin and Hartree, however, contains some obscure points. For example, they do not explain the apparent development of the band at 431 mμ from that at 448 mμ, not only on treatment with carbon monoxide, but also on addition of cyanide. They also give no information about the position of the main band of cytochrome *a* which appears to be missing although the α-band of cytochrome *a* is present. Probably no further progress will be made in this direction unless the qualitative spectroscopic experiments are replaced by quantitative investigations.

† D. Keilin and E. F. Hartree, *Proc. Royal Soc.* B **127**, 167 (1939).

‡ O. Warburg and E. Negelein, *Bioch. Zeitschr.* **262**, 237 (1933); **266**, 1 (1933).

§ Id., ibid. **233**, 486 (1931); **238**, 135 (1931).

CHAPTER XV

THE CHEMICAL CONSTITUTION OF THE OXYGEN TRANSPORTING HAEM

A COMPARISON is made of the spectrum of the oxygen transporting enzyme in yeast or in acetic acid bacteria with that of haemoglobin in the following table:

	Main band	*α-band*
Carbonyl haemoglobin	420 mμ	570 mμ
Carbon monoxide enzyme	431 mμ	591 mμ

It can be seen that the absorption bands of the enzyme occur at considerably higher wave-lengths. These differences are not due to the protein components, for by coupling the protohaemin with bases the spectrum can be displaced a few mμ towards the red, but in no case to the extent of 20 mμ which is the amount by which the α-bands of the enzyme and haemoglobin differ.

For this reason we were convinced that the enzyme haems are different from protohaem. In 1930 we started experiments to prepare haemins which would behave spectroscopically like the enzyme haemins.

Our starting materials were protohaemin and chlorophyll. The methods we used were essentially those of Hans Fischer,† and Fischer's haemin and chlorophyll formulae made it possible for us to obtain information concerning the chemical constitution of the enzyme haems from a comparison of the spectra.

1. Classification of the haemins according to colour‡

We recognize red, green, and dichroic haemins. Red haemins for example, include protohaemin, mesohaemin, and deuterohaemin. Coproporphyrin discovered in animal bodies by Hans Fischer gives an iron compound which is also a red haemin. Other red haemins are pyrrohaemin, phyllohaemin and rhodo-

† Hans Fischer, Hans Orth, and Adolf Stern, *Die Chemie des Pyrrols*, Leipzig, 1934 to 1940.

‡ O. Warburg and E. Negelein, *Bioch. Zeitschr.* **244**, 9 (1932).

haemin, whose porphyrins were prepared by Willstätter from chlorophyll. The red haems have the following bands:

	Main band	*α-band*
Carbon monoxide compounds of the red haems	420 $m\mu$ and at shorter wavelength	570 $m\mu$ and at shorter wavelength

Up till now no oxygen transporting enzyme has been found, the haemin of which could be a red haemin as judged from the position of its bands.

Green haemins can be obtained by oxidation of red haemins. The green haemins obtainable from chlorophyll by replacing the magnesium by iron are however, more important. Whilst the α-bands of the red haemins lie in the green, the green haemins have their α-bands in the red. Oxygen transporting enzymes having a green haemin are found in bacteria. For example, the oxygen transporting haemin of Azotobacter† is a green haemin, the α-band of which is in the red at 632 $m\mu$. This is displaced by carbon monoxide to 637 $m\mu$.

Intermediate between the red and the green haemins we have dichroic haemins, solutions of which appear red or green according to their concentration and the thickness of the layer through which they are viewed. Their α-bands lie in the yellow, i.e. between the α-bands of the red and the green haemins.

Oxygen transporting enzymes having a dichroic haemin are widely distributed in nature. The oxygen transporting haemin of yeast, of the acetic acid bacteria, of the retina and probably of most aerobic cells is a dichroic haemin.

All three types of haemins, red, green and dichroic have the property of transporting oxygen catalytically by a valency change in their iron atoms.‡

The dichroic haemins which we have investigated show the particular property of readily changing by a reversible reaction into red haemins. This phenomenon, which we discovered in

† Cf. Chap. XIV.

‡ O. Warburg and F. Kubowitz, *Bioch. Zeitschr.* **227**, 184 (1930).

1930,† is probably of physiological importance, but the chemistry is not as yet completely understood.

If, for example, a dichroic haemin is dissolved in a weakly alkaline cysteine solution at room temperature, in the course of an hour a red haemin is formed, and from a solution of the latter the original dichroic haemin can be precipitated on acidification. Or, if a dichroic haemin is dissolved in methyl alcohol containing a little hydrochloric acid, it changes to a red haemin, from a solution of which the original haemin is obtained on concentrating. The dichroic and the green haemins, but not the red haemins, also possess the property, noteworthy for physiological reasons, of being able to oxidize catalytically carbon monoxide to carbon dioxide at room temperature.‡

The green haemins, and still more so the dichroic haemins, are therefore more reactive than the red haemins.

2. Preparation of a green haemin from a red haemin§

If protohaemin is dissolved in aqueous pyridine containing hydrazine, and oxygen is passed through the solution, in a few minutes the red colour changes to green. The green haemin which is isolated from the solution in an amorphous state gives a crystalline green ester on heating with methyl alcoholic hydrogen chloride. This compound, in contrast to the green haemin from which it is isolated, shows no characteristic absorption bands.

This was our first attempt to prepare a haemin with the spectroscopic properties of the enzyme haemin. The attempt was a complete failure. Neither of the two green iron compounds possessed any property of catalytic oxidation.

R. Lemberg‖ showed later that the green crystalline ester is an iron salt of a biliverdin ester, the first crystalline bile pigment

† O. Warburg, E. Negelein, and E. Haas, *Bioch. Zeitschr.* **227**, 171 (1930); O. Warburg and E. Negelein, ibid. **244**, 9 (1932); O. Warburg and W. Christian, ibid. **235**, 240 (1931); E. Negelein, ibid. **266**, 412 (1933).

‡ Id., ibid. **243**, 386 (1931).

§ O. Warburg and E. Negelein, *Chem. Berichte*, **63**, 1816 (1930).

‖ R. Lemberg, *Bioch. Journal*, **29**, 1322 (1935).

obtained artificially from the blood pigment. Since that time† our oxidation reaction has been applied with success to many haemins and has thus been used for a purpose which was not foreseen.

Lemberg and Wyndham‡ are of the opinion that the green haemins obtained from the red haemins by oxidation can act in cells as oxygen transporting haemins, and in proof of this they relate them to the higher wave-length bands which we discovered in azotobacter and acetic bacteria.§ In contrast to this we take the view that the naturally occurring oxygen transporting green haemins are more closely related to the chlorophyll haemins.

Information as to the chemical changes involved in the formation of the green haemins by oxidation of the red haemins is available from the work of Stier from Hans Fischer's Institute. According to Stier the oxidation takes place at the methine bridge joining the pyrrole nuclei I and II. The CH is first oxidized to COH without any fission of the porphyrin ring. The ring is opened only after warming with methyl alcoholic hydrogen chloride according to our method for obtaining the green ester, and the bile pigment is then formed. In my opinion the spectroscopic findings appear to fit this theory of Stier.

3. Preparation of a green haemin from chlorophyll *b*

If the magnesium is removed from chlorophyll and the phytyl ester group hydrolysed, leaving the carboxyl group, there are formed crystalline substances, discovered by Willstätter‖ and called by him phaeophorbides. Phaeophorbide *a* is formed from chlorophyll *a* and phaeophorbide *b* from chlorophyll *b*.

Hans Fischer and Bäumler†† showed that iron can be introduced into the phaeophorbide. The iron salt of phaeophorbide *a* was the first green haemin to be prepared from chlorophyll. It has remarkable catalytic properties.‡‡ When brought into contact with nuclei-free red blood cells, the oxygen requirement

† E. Stier, *Z. f. physiologische Chem.* **255**, 209 (1938); **272**, 239; **274**, 231; **275**, 155 (1942).

‡ R. Lemberg and R. Wyndham, *Proc. Royal Soc. New South Wales*, **70**, 343 (1936).

§ Cf. Chap. XIV.

‖ R. Willstätter and A. Stoll, *Untersuchungen über Chlorophyll*. Berlin, 1913.

†† H. Fischer and R. Bäumler, *Liebigs Ann.* **474**, 65 (1929).

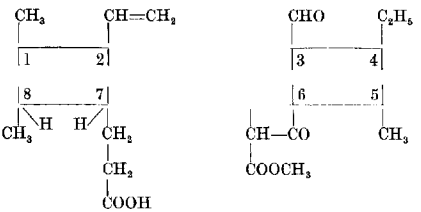
‡‡ O. Warburg and F. Kubowitz, *Bioch. Zeitschr.* **227**, 184 (1930).

is increased 20 times. This increased respiration can be inhibited by carbon monoxide since the ferrous form of the green haemin combines with carbon monoxide.

The corresponding haemin of chlorophyll *b* was prepared by us in 1931,† and obtained crystalline as the propionic acid compound. We coupled this green haemin with globin and so obtained a green haemoglobin‡ the carbon monoxide compound of which has a band in the red at 630 mμ.

The band of the carbon monoxide compound of the oxygen transporting enzyme of azotobacter§ lies at 637 mμ. A relationship with the green haemin from chlorophyll *b* would appear to be indicated.

According to Fischer‖ the green haemin from chlorophyll *b* has the following constitution in so far as the substituents on the four pyrrole rings are concerned



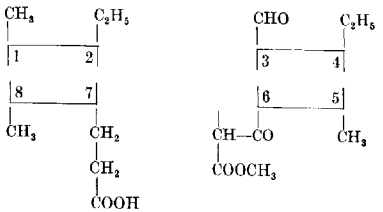
Green haemin from chlorophyll *b*

4. Dichroic haemin from chlorophyll *b*

When phaeophorbides are warmed at 50° for a few minutes with hydrogen iodide in acetic acid, there are formed, as was discovered by Hans Fischer,†† several porphyrins which have been of importance in the chemistry of chlorophyll and which were called phaeoporphyrins by their discoverer. In this reaction, the vinyl group is reduced to an ethyl group, the hydrogen at 7 and 8 being oxidized. The reaction is neither an oxidation nor a reduction, but an isomeric change.

† O. Warburg, *Chem. Berichte*, **64**, 682 (1931).
‡ O. Warburg and E. Negelein, *Bioch. Zeitschr.* **244**, 9 (1932).
§ E. Negelein and W. Gerischer, ibid. **268**, 1 (1934).
‖ Hans Fischer, *Die Chemie des Pyrrols*, Leipzig, 1934 to 1940.
†† Id., *Berichte der Bayer. Akademie der Wissenschaften*, 1919, p. 77.

We have applied the method to phaeophorbide *b* and have obtained phaeoporphyrin *b*.† On introducing iron, phaeohaemin *b* was formed, this being crystallized as its propionic acid derivative to which, according to Hans Fischer, the following formula may be given:



Phaeohaemin *b*

Phaeohaemin *b* is a dichroic haemin and is converted to a red haemin in cysteine solution,‡ this being due, as Hans Fischer§ showed, to the addition of cysteine to the formyl group. Reconversion to the dichroic haemin on acidification of the solution of the red haemin would then be brought about by the removal of the cysteine.

Phaeohaemin combines with globin to give a phaeohaemoglobin‖ which is a dichroic haemoglobin and which has the interesting property of reacting reversibly not only with carbon monoxide, but also with oxygen.

A comparison of the spectrum of this dichroic haemoglobin with the red haemoglobin of blood and with the oxygen transporting enzyme is shown below:

	Main band	*α-band*
Carbon monoxide protohaemoglobin . .	420 mμ	570 mμ
Carbon monoxide enzyme of yeast . .	431 mμ	591 mμ
Carbon monoxide phaeohaemoglobin. .	435 mμ	600 mμ

In so far as its spectrum is concerned the enzyme therefore comes between the two haemoglobins, but is closer to phaeohaemoglobin than to protohaemoglobin.

† O. Warburg and W. Christian, *Bioch. Zeitschr.* **235**, 240 (1931).
‡ Section 1 of this chapter. § Hans Fischer, *Chemie des Pyrrols.*
‖ O. Warburg and E. Negelein, *Bioch. Zeitschr.* **244**, 9 (1932).

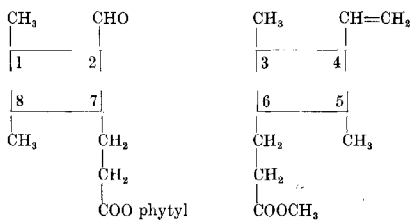
5. The haemin of Spirographis

The blood of Spirographis, which appears green in thin layers and red in thick layers, contains a pigment which, like haemoglobin, forms dissociating oxygen- and carbon monoxide-compounds. Ray Lankaster,† who discovered the pigment, named it chlorocruorin. Munroe H. Fox‡ found that on evaporating a drop of blood with acetic acid and sodium chloride the chlorocruorin crystallized out.

We have isolated and crystallized the haemin in sufficiently large amounts to permit its analysis and chemical investigation.§ We also obtained the porphyrin and some of its derivatives in crystalline form.

Our chemical investigation showed that the Spirographis haemin contains less carbon but more oxygen than protohaemin. Five atoms of oxygen are present, of which four are in carboxyl groups whilst the fifth reacts with hydroxylamine. Moreover, the Spirographis haemin has no, or at most one, vinyl group.

For the conclusive investigation and proof of the constitution we are indebted to Hans Fischer‖ who has put forward the following formula:



The Spirographis haemin

† Ray Lankaster, *Journ. Anat. and Physiology*, **2**, 114 (1868); **4**, 119 (1870). MacMunn, *Quarterly Journ. of Microsc. Science*, **25**, 469 (1885). A. B. Griffith, *Compt. Rend. Paris*, **114**, 1277 (1892).

‡ Munroe H. Fox, *Proc. Cambridge Philos. Soc. Biolog. Science*, **1**, 204 (1924); *Proc. Royal Soc. London*, B **99**, 199 (1926).

§ O. Warburg, E. Negelein, and E. Haas, *Bioch. Zeitschr.* **227**, 171 (1930); O. Warburg and E. Negelein, ibid. **244**, 239 (1932).

‖ Hans Fischer and C. v. Seemann, *Zeitschr. f. physiolog. Chem.* **242**, 133 (1936); Hans Fischer and G. Wecker, ibid. **272**, 1 (1941).

The Spirographis haemin is therefore protohaemin in which a vinyl group has been oxidized to a formyl group.

It is a dichroic haemin and can easily be converted into a red haemin, for example, by allowing it to stand for some hours in a weakly alkaline solution of cysteine. On acidification, the dichroic haemin is again precipitated. As in the case of phaeohaemin, Hans Fischer explained this reaction by the addition to the formyl group of cysteine and its subsequent removal on acidification.

By coupling the Spirographis haemin with globin we obtained Spirographis haemoglobin,† the bands of which occur at shorter wave-lengths than those of chlorocruorin, but at longer wave-lengths than the protohaemoglobin bands. Below is a comparison of these bands with those of the oxygen transporting enzyme in yeast, and it can be seen that the enzyme bands lie near those of the Spirographis haemoglobin:

	Main band	*α-band*
Carbon monoxide protohaemoglobin . .	420 mμ	570 mμ
Carbon monoxide enzyme . . .	431 mμ	591 mμ
Carbon monoxide spirographis haemoglobin	435 mμ	590 mμ
Carbon monoxide chlorocruorin . .	440 mμ	602 mμ

6. Dichroic haemin from heart muscle‡

When minced horse heart muscle is washed with water till the wash water is colourless, haemoglobin, myoglobin, and a part of the cytochrome *c* are removed. The residue contains the water-insoluble part of cytochrome *c*, the whole of cytochromes *a* and *b*, and the oxygen transporting enzyme.

Acetone containing hydrochloric acid extracts from the residue two haemins: protohaemin derived from cytochromes *b* and *c*, and a dichroic haemin derived from cytochrome *a* and the oxygen transporting enzyme. The haemochromogen reaction with pyridine and hydrosulphite gives a strong band at 557 mμ and a considerably weaker one at 587 mμ.

† O. Warburg and E. Negelein, *Bioch. Zeitschr.* **244**, 9 (1932).
‡ E. Negelein, ibid. **266**, 412 (1933).

We separated the dichroic haemin from the red haemin and crystallized it as the pyridine haemochromogen. For analysis, the haematin, which did not crystallize, was prepared, and gave 6·5 per cent. iron, and 4 nitrogen atoms per iron atom. Protohaematin contains 8·6 per cent. iron. It follows from the low iron content of the dichroic haematin that it contains an additional part of molecular weight 300. This addendum must be free from nitrogen since the total nitrogen of the haematin is pyrrole nitrogen.

The first thought that arises is that one of the two carboxyl groups might be esterified with a high molecular weight alcohol, and this would have been in agreement with the behaviour of the haemin towards organic solvents. Esterification with phytol, the molecular weight of which is 296, would lower the iron content of the Spirographis haemin from 8·6 to 6·0 per cent., whilst 6·5 per cent. of iron had been found.

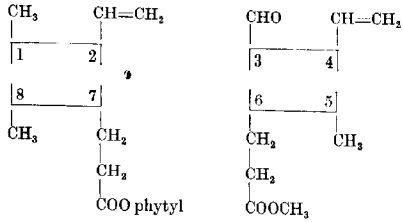
Like phaeohaemin *b* and Spirographis haemin, the haemin from heart muscle also has the property of being readily converted into a red haemin and back again into a dichroic haemin. If, as Hans Fischer believes, this phenomenon depends on the presence of a formyl group, then the dichroic haemin from heart muscle would also contain such a group.

7. Chemical constitution of the enzyme haemin

Since all aerobic cells respire, but do not all contain haemoglobin or chlorophyll, the oxygen transporting enzyme must have been of earlier evolution than either haemoglobin or chlorophyll. It does not follow from this, however, that haemoglobin and chlorophyll have been developed from the oxygen transporting enzyme, but it is probable that they have.

The problem then arises as to what must be the constitution of a haemin with the light absorption properties of the enzyme haemin if it is the forerunner of both haemoglobin and chlorophyll.

A formula such as the following would provide a simple solution to this problem.



Oxygen transporting enzyme haemin ?

If the formyl group of this compound were reduced to a methyl group and the ester groups hydrolysed, protohaemin would result. If the vinyl group in position 4 was reduced to an ethyl group, the propionic group at 6 oxidized to the ketonic acid, and the two H atoms allowed to wander to 7 and 8, then we should have the haemin of chlorophyll *b*. Further, if the formyl group was reduced to a methyl group, we should have the haemin of chlorophyll *a*.

In order to verify this theory the oxygen transporting haemin would have to be isolated and this might not be so difficult today. A suitable starting material might be acetic acid bacteria, because they contain no cytochrome *a*. A dichroic haemin obtained from this source could only be the haemin of the oxygen transporting enzyme, whilst the dichroic haemin isolated from yeast or heart muscle would be derived mainly from cytochrome *a*.

Note. These views on the constitution of the haemin of the oxygen transporting enzyme were written in 1944 in the hope that Hans Fischer would quickly prove or disprove their validity. This hope is now gone, and no one in the immediate future, as far as can be seen, will be capable of solving this problem. The often repeated saying that no one is irreplaceable does not apply in Science.

CHAPTER XVI

THE OXYGEN TRANSPORTING HAEM

THE discussions which took place with Willstätter, Wieland and Euler about oxygen transporting iron began in 1922 and ended in 1928. They were brought to an end by a controversy with Keilin which started in 1929 and finished in 1939.

Keilin certainly admitted that the oxygen transporting enzyme oxidized the cytochrome, but he disputed the view that the oxygen transporting enzyme was a haem compound or indeed an iron compound of any kind. This in spite of the fact that in 1929 at the latest the haem nature of the oxygen transporting enzyme had been proved. The discussions with Keilin also were therefore of a negative character.

1. The name of the oxygen transporting enzyme

The name of the enzyme played an essential part in these discussions. Keilin changed the name of the enzyme several times. I should therefore like to make the following observations:

The nature of the autoxidizable part of living matter, that is, the part that reacts with oxygen, has been since the time of Lavoisier the main problem of respiration, and in order to give expression to the solution of the problem in the name of the enzyme, 'oxygen transporting' enzyme seemed an appropriate title. I admit that 'enzyme reacting with oxygen' might be more correct, since the enzyme does not give up the oxygen as such, but rather as its equivalent in oxidizing action. But by the explanation of the transport as being due to a valency change ferrous $\rightleftharpoons$ ferric, any misinterpretation of the transport was excluded.

After 1934, but not earlier, the enzyme could have been differently named. For in 1934 we found that the ferric iron of the enzyme mainly oxidized ferrous cytochrome. From then on the enzyme could have been named 'cytochrome oxidase', and one of the two substrates with which the enzyme reacted,

namely, the cytochrome would thereby have been given preference over the other.

I have for several reasons kept to the old name. One reason is that everybody knows what oxygen is, whilst the idea of cytochrome is known only to the initiated, and often not even to them on account of the changing definition.

Again it must be remembered in this connexion that the right to name a substance is reserved for its discoverer. All names are disputable no matter whether they are concerned with a subject, a substance, or a person. What might happen, however, if every name that was objectionable for some reason or other were changed can be appreciated by a consideration of examples such as lactic acid or succinic acid. Those names are certainly more controvertible than 'histohaemin' or 'oxygen transporting enzyme'.

However, in the chemical nomenclature of enzymes† I have always designated the oxygen transporting enzyme as

$$\text{Ironprotcid } O_{2\text{-}\,\text{Cytochrome } a}$$

in which neither of the two substrates is given preference over the other.

2. Indophenol oxidase‡

In 1928 Keilin repeated our experiments on the carbon monoxide inhibition of respiration, with the modification that he replaced the natural substrates with a mixture of α-naphthol and *p*-phenylenediamine which is oxidized *in vivo* to indophenol blue.

Keilin proved that the oxidation of this unnatural substrate was inhibited by carbon monoxide like that of the natural substrate, and that the inhibition was reversed on exposure to light.

Such a result was only to be expected, since it was known that the carbon monoxide does not react with the substrates, but

† O. Warburg, *Ergebnisse der Enzymforschung*, **7**, 210 (1938).
‡ D. Keilin, *Proc. Royal Soc. London*, B **104**, 206 (1929).

with the iron of the enzyme, and that the oxygen transporting enzyme and the substrate in the chain†

$$O_2 \rightarrow \text{enzyme} \rightarrow \text{cytochrome} \rightarrow \ldots \text{substrate}$$

do not come into contact with one another. Why therefore should the action of carbon monoxide on the oxygen transport be affected by changing the substrate?

Keilin, however, regarded his result as unexpected and important enough to justify changing the name of the oxygen transporting enzyme to 'indophenol oxidase', and he found much support for this. For nine years, from 1929 to 1938, the oxygen transporting enzyme took on what might be almost called the absurd name 'indophenol oxidase'. The importance of the absorption spectrum was even lessened by the change of name:‡ 'In short, the properties of the respiratory enzyme are not those of a haematin compound, but those of the indophenol oxidase.'

In 1938 Keilin§ again started up his experiments on the indophenol blue formation. He found, however, this time that it was not the oxygen transporting enzyme, but the cytochrome which oxidized the naphthol-diamine mixture to the indophenol pigment. That is, he showed that our previously published scheme of 1927 applied also for this unnatural substrate.

In spite of Keilin's principles in regard to nomenclature, he did not find it necessary to rename the cytochrome. Instead he again changed the name of the oxygen transporting enzyme. 'Indophenol oxidase' was incorrect. 'The correct name of this oxidase is . . . Cytochrome oxidase.'

Thus indophenol oxidase disappeared from the literature after having been dramatically introduced into Science where it remained for nine years without much justification.

3. Chemical constitution

Keilin put forward the following arguments‖ against the

† Section 10, Chap. VIII.
‡ M. Dixon, *Biological Reviews*, **4**, 352 (1929) [p. 387].
§ D. Keilin, *Proc. Royal Soc. London*, B **125**, 171 (1938).
‖ Id., ibid., B **104**, 206 (1929).

view that the oxygen transporting enzyme was a haem compound as we had shown it to be from the reaction spectrum:

1. Haem catalysis could not be inhibited by cyanide.
2. Haem catalysis could not be inhibited by carbon monoxide.
3. The photochemical reaction spectrum of the carbon monoxide inhibition was not the spectrum of the oxygen transporting enzyme, but that of cytochrome.

4. Experiments with hydrogen cyanide

Keilin added cyanide to haemochromogen solutions, and when these were shaken with oxygen he found that the oxidation of the ferrous iron to the ferric state was not inhibited. Since respiration was inhibited by cyanide, he concluded that the oxygen transporting enzyme could not be a haem compound.

He overlooked the fact that in oxygen transport by iron two oxidation stages of the iron are involved—the divalent and the trivalent, and that we had shown that it is the trivalent and not the divalent iron of the oxygen transporting enzyme which combines with the cyanide. Cyanide does not inhibit the oxidation of ferrous iron, but rather the reduction of ferric iron.†

5. Experiments with carbon monoxide

Keilin shook haemochromogen solutions with mixtures of carbon monoxide and oxygen and found that at the end of the reaction the whole of the iron was present in the ferric state. No trace of a carbon monoxide-ferrous compound could be found. Since, however, the oxygen transporting enzyme in the respiring cell partitioned itself between oxygen and carbon monoxide, Keilin concluded that it could not be a haem compound.

He overlooked the fact that ferric iron in the respiring cell is always reduced back again to ferrous iron, and that the oxygen and the carbon monoxide compete for the ferrous iron thus formed. Actually, the partition equation of haemoglobin, as was shown at the time by its detailed derivation, was based on

† O. Warburg, *Bioch. Zeitschr.* **189**, 354 (1927) [p. 372].

mathematical theory. Besides this, however, we had shown purely empirically in model experiments with oxygen transporting haemins, that the haem iron partitioned itself between carbon monoxide and oxygen just like the oxygen transporting enzyme in respiring cells.†

6. Reaction spectrum

After Keilin had proved by his experiments with cyanide and carbon monoxide that the oxygen transporting enzyme could not be a haem compound, he had to explain why, in spite of this, the photochemical reaction spectrum of the carbon monoxide inhibited respiration was a haem spectrum.

To do this he assumed that the light absorbing substance in respiring cells was not the oxygen transporting enzyme but cytochrome, which, acting as a photochemical sensitizer, transported the light energy to the enzyme. If it were possible, said Keilin, to isolate the oxygen transporting enzyme and to determine the photochemical spectrum—which would, however, then be unnecessary—then it would be permissible to relate the reaction spectrum to the oxygen transporting enzyme. But 'unfortunately the effect of light can be studied only on cells already containing four other haematin compounds'.

This was an objection which could be logically admitted although it was unsupported experimentally and was only invented for purposes of argument. That cytochrome, which does not fluoresce, might act as a photochemical sensitizer was, however, extremely improbable. Moreover, in our model experiments‡ it was shown that only such light as is absorbed by the carbon monoxide compound causes dissociation of the carbon monoxide from the iron. In a mixture of haem and carbon monoxide-haem, the light which is absorbed by the haem is photochemically inactive.

Then in 1932 Keilin's objection was experimentally refuted. The reaction spectrum was so accurately determined§ that any

† H. A. Krebs, *Bioch. Zeitschr.* **193**, 347 (1928); **204**, 322 (1929).
‡ O. Warburg and E. Negelein, ibid. **200**, 414 (1928) [pp. 453, 458].
§ F. Kubowitz and E. Haas, ibid. **255**, 247 (1932).

question of identity with the cytochrome spectrum was excluded, whilst according to Keilin's theory the reaction spectrum should have been the cytochrome spectrum.

7. Phenol oxidase

In 1937 we isolated phenol oxidase,† the oxygen transporting enzyme of potato tubers, and found that it is a copper proteid which transports oxygen by virtue of the reaction cuprous $\rightleftharpoons$ cupric.

At once Keilin‡ put forward the theory that the oxygen transporting enzyme of aerobic cells was a copper proteid. In his view the function of the oxygen transporting enzyme as 'cytochrome oxidase', formerly obscure, now became understandable, since cytochrome could be oxidized with copper sulphate, but not with ferric sulphate.

The first argument against Keilin's copper theory was that the carbon monoxide inhibition of phenol oxidase is not sensitive to light, and that no light sensitive carbon monoxide-copper compounds had previously been found. Keilin invoked again in this case his sensitization theory of 1929. According to this, the light absorbing substance in respiring cells was cytochrome. The absorbed light energy was given up by the cytochrome to the oxygen transporting copper and its carbon monoxide compound so that dissociation of the carbon monoxide from the copper followed.

This theory would only have been of importance if Keilin had been able to support it experimentally, i.e. if he had been able to show that the carbon monoxide inhibition of phenol oxidase could be made light sensitive when haemin was added to the enzyme solution. Otherwise it was again only an objection devised for purposes of argument, an objection that was already obsolete in 1938, since it had been known six years previously that the reaction spectrum was not the cytochrome spectrum.

Besides this, in 1938, not the whole of the cytochrome but only the *a*-component was supposed to act as an optical

† F. Kubowitz, ibid. **292**, 221 (1937).
‡ D. Keilin, *Nature*, **141**, 870 (1938).

sensitizer, for it was only in cells which contained cytochrome *a* that the carbon monoxide inhibition of respiration was supposed to be light sensitive. In point of fact, however, acetic acid bacteria which contain no cytochrome *a* had been our main material for the determination of the optical reaction spectrum.

8. Cytochrome

If one considers that we had either to answer Keilin's objections in continuous discussions, or stand aside and watch how all that had been explained by painstaking experimentation was in danger of being misunderstood, it can be readily appreciated how pleased we were when Keilin in 1939 became reconciled to the idea of the oxygen-transporting haem. He wrote in 1939 that he had discovered by spectroscopic examination of heart muscle preparation a new autoxidizable haem compound, the ferrous form of which reacted with carbon monoxide and the ferric form with cyanide, and the spectrum of which corresponded with the photochemical reaction spectrum of the carbon monoxide inhibited respiration. Probably the new haem compound, which received the name cytochrome a_3, was identical with the haem of the oxygen transporting enzyme.†

† D. Keilin and E. F. Hartree, *Proc. Royal Soc. London*, B **127**, 167 (1939). Cf. also section 3, Chapter XIV.

CHAPTER XVII

COPPER, THE OXYGEN TRANSPORTING CONSTITUENT OF PHENOL OXIDASE

PHENOL oxidase is an oxidizing enzyme occurring in plants and capable of transporting molecular oxygen to phenols. Raper† found that ortho-quinones are formed by its action. According to the conditions under which the oxidase reacts the *o*-quinones are further oxidized with formation of dark-coloured substances, or they are again reduced back to phenols by the reducing substances in the cell. *In vivo*, the phenols are not substrates, but rather links in a chain of catalysts:

$O_2 \rightarrow$ oxidase $\rightarrow$ catechol $\rightarrow$ *o*-quinone $\rightarrow$ . . . substrate.

The phenol oxidase is different from the oxygen transporting iron proteid in being soluble in water. It was therefore possible to isolate the enzyme, and to study in stoichiometric experiments how the enzyme transports oxygen and why it is inactivated by cyanide and by carbon monoxide. In this way, using the methods of analytical chemistry, what had been predicted was proved to be the case, namely, that the enzyme was a heavy metal compound, since it was inhibited by cyanide and carbon monoxide, and that it could not be an iron compound because the inhibition by carbon monoxide was not light sensitive.

1. Isolation‡

We isolated phenol oxidase from potatoes in 1937 and found that it was a copper proteid containing about 0·2 per cent. copper.

For the success of this work two manometric methods were required: a manometric test to show that the extent of the action was proportional to the enzyme concentration, and a manometric method of determining very small amounts of copper.

† H. S. Raper, *Ergebnisse der Enzymforschung*, **1**, 270 (1932).

‡ F. Kubowitz, *Bioch. Zeitschr.* **292**, 221 (1937); **299**, 32 (1938).

Tests which depend on the determination of the oxidation products of the phenols are not trustworthy, since these products bring about secondary oxidations, and if they accumulate they destroy the enzyme. The substrate we used was the Robison ester, to a solution of which some specific dehydrogenase and a very small amount of catechol were added. When we introduced phenol oxidase into the mixture, the catechol was oxidized to *o*-quinone which, however, was immediately reduced back by the dihydro-pyridine nucleotide to catechol. Under these conditions the amount of oxygen taken up per minute was proportional to the concentration of phenol oxidase.

In the manometric copper determination,† the main compartment of a manometric vessel contained alkaline pyrophosphate solution, and the side tube acid cysteine solution and the enzyme, the copper content of which had to be determined. The enzyme was decomposed by the acid in the tube to copper salt and denatured protein. When the contents of the side tube were allowed to run into the main compartment, the copper salt transported oxygen to the cysteine, and the rate of this reaction gave the copper content of the enzyme preparation. The method is about 200 times more sensitive than the most accurate colorimetric copper determination, and it has the further advantage that ashing of the enzyme is not necessary.

Using these two methods it was discovered that the activity of phenol oxidase is proportional to the copper content. When the activity is plotted graphically as a function of the copper content a straight line is obtained which passes through the axis. That is, for a copper content of zero, the activity is also zero (Fig. 27).

2. Absorption spectrum

Although phenol oxidase is a complex heavy metal compound, it has no characteristic absorption bands. Strong solutions have a pale yellow colour; spectroscopic examination of these

† O. Warburg, *Bioch. Zeitschr.* **187**, 255 (1927). Also section 9, Chapter V. The gas space in the manometer vessel should contain pure oxygen, not air.

reveals a protein band at 275 mμ, but no bands in the visible or in the long-wave ultra-violet region.

3. Decomposition and resynthesis

When a solution of phenol oxidase is dialysed against water, the activity and the copper content remain constant. The copper

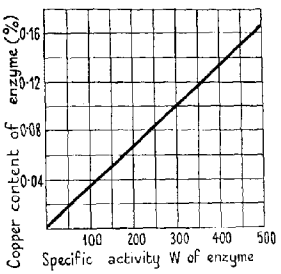


FIG. 27. Specific activity and copper content of potato oxidase.

is, therefore, so firmly bound that it cannot be removed by dialysis against water.

When the dialysis is carried out against N/100 hydrogen cyanide, however, the copper dialyses out and the activity disappears with the copper. Cyanide therefore decomposes the enzyme into protein and copper salt.

The protein remains in solution and is not denatured. When the hydrogen cyanide is removed by dialysis against water and copper sulphate is added to the solution, the full activity of the enzyme is regained, and the copper is again not removable by dialysis against water. The yield in the decomposition and resynthesis is about 100 per cent.

In such experiments, which in other respects are simple to carry out, it is necessary to take care that the test solutions are as free as possible from traces of copper impurities. If the protein which is being tested amounts to 5 γ, for example, the equivalent amount of copper is 0·01 γ. This is roughly the amount of copper which must be removed from the test volume amounting to 3 c.c. Otherwise, enzyme activity is always

found, even if the enzyme protein itself is completely copper free.

4. Mechanism of catalysis and anticatalysis

When phenol oxidase is dialysed against solutions which are saturated with carbon monoxide, the copper content of the enzyme is not decreased. Carbon monoxide, therefore, does not inhibit the enzyme as cyanide does by splitting off the copper, but by combining with the enzyme. Further information about this combination is given by the following experiment:

The gas space of a manometric vessel is filled with carbon monoxide and the main compartment contains a solution of phenol oxidase. One of the two side tubes of the vessel holds catechol, the other cyanide.

Enzyme which is not in contact with the substrate does not take up any carbon monoxide. If, however, catechol is added from the side tube, carbon monoxide is taken up and to the extent of one molecule for every two atoms of copper.

If cyanide is added from the side tube when the uptake of carbon monoxide has ceased, the carbon monoxide is again set free into the gas space. Thus it is proved that it is the copper of the enzyme which combines with the carbon monoxide, because hydrogen cyanide which is known to remove the copper frees the carbon monoxide from its combination. Also it is proved that it is monovalent copper in the enzyme which binds the carbon monoxide, because combination with carbon monoxide only takes place after the addition of catechol.

By means of this stoichiometric experiment the action of phenol oxidase and its inhibition by cyanide or carbon monoxide is shown to be due to the action of the copper. The enzyme reacts by virtue of the valency change cuprous $\rightleftharpoons$ cupric. Cyanide inhibits the action because it splits off copper from the protein, carbon monoxide inhibits the action by combining with the monovalent copper.

Since in the carbon monoxide compound of the enzyme we have one molecule of carbon monoxide combined with two atoms of copper, it is probable that the reacting unit of the

enzyme contains two atoms of copper and that the equation for the phenol oxidation is:

$$1\ (Cu^{++})_2 + 1\ \text{catechol} = 1\ (Cu^{+})_2 + 1\ o\text{-quinone} + 2H^{+}.$$

5. Substrate

In order to test whether one substance only is oxidized by the enzyme we added to the main compartment of a manometer vessel about 1 mg. of the substance to be tested dissolved in neutral phosphate. The gas space contained oxygen, the side tube a few γ of the pure enzyme. When the enzyme was added at time t_0 to the substrate from the side tube, the absorption of oxygen usually started at once if the substance was oxidized, but in some cases only after an induction period.

Catechol is oxidized most quickly of all substances. Pyrogallol, 3:4-dihydroxyphenylalanine, dihydroxycinnamic acid, protocatechuic acid, tyrosine, are all oxidized about 1,000 times more slowly than catechol. After an induction period, phenol and *p*-cresol are oxidized. Resorcinol, hydroquinone, and ascorbic acid are not oxidized at all.

The induction period in the oxidation of monophenols observed by M. Graubart and J. M. Nelson,† with crude enzyme solutions can be shortened by the addition of a very small amount of catechol.‡ Again, such substances as hydroquinone and ascorbic acid, which do not appear to be oxidized in the absence of catechol, are oxidized when small amounts of the latter are added. Resorcinol, however, even in the presence of catechol is not oxidized.

Probably in the case of monophenols, *o*-diphenols are first formed by a slow reaction, and these then act catalytically by virtue of the change diphenol $\rightleftharpoons$ quinone. It may be assumed that the same reversible change takes place when added catechol reacts catalytically as in the case of hydroquinone or ascorbic acid.

When crude plant extracts are used instead of the purified enzyme the phenomena are less marked because plant cells

† M. Graubart and J. M. Nelson, *Journ. biolog. Chem.* **111**, 761 (1935).

‡ C. E. Pugh, *Bioch. Journ.* **24**, 1442 (1930).

contain substances, probably *o*-diphenols, which shorten the induction period. If, therefore, in the course of the isolation of the enzyme, oxidation reactions, which were shown by the crude extracts, disappear, it cannot be concluded that the crude extracts contain various phenol oxidases such as a mono- and a diphenol oxidase.

6. Reactivity of the copper

With catechol as substrate the rate at which the divalent copper in the enzyme reacts is

$$-\frac{d\,\text{cupric}}{dt} = k_{\text{red}} \times [\text{cupric}] \times [\text{catechol}],$$

and the rate at which the monovalent copper of the enzyme is reoxidized by molecular oxygen is

$$-\frac{d\,\text{cuprous}}{dt} = k_{\text{ox}} \times [\text{cuprous}] \times [\text{O}_2],$$

where k_{red} and k_{ox} are the velocity constants of the reduction and oxidation. Expressing the concentrations in moles/litre we found at 25°†

$$k_{\text{red}} = 0{\cdot}59 \times 10^8 \left[\frac{1}{\text{minutes}\,\frac{\text{moles catechol}}{\text{litre}}}\right]$$

$$k_{\text{ox}} = 1{\cdot}42 \times 10^8 \left[\frac{1}{\text{minutes}\,\frac{\text{moles O}_2}{\text{litre}}}\right].$$

When the concentration of the catechol is high during the oxygen transport, the total copper of the enzyme is in the monovalent form, and the rate of the transport is determined by k_{ox} and the oxygen concentration. On saturating the enzyme solution with oxygen at atmospheric pressure and at 25°, with $k_{\text{ox}} = 1{\cdot}42 \times 10^8$ we find, therefore, that the enzyme can transport a maximum of

$1{\cdot}55 \times 10^7$ c.mm. oxygen per minute and per mg. copper.

† O. Warburg, *Ergebnisse der Enzymforschung*, **7**, 210 (1938).

7. Combined action with other enzymes

We have found that *o*-quinone reacts rapidly with dihydropyridine nucleotide according to the equation

o-quinone + dihydropyridine nucleotide
= catechol + pyridine nucleotide.

When, therefore, phenol oxidase and catechol are added to a pyridine proteid and its specific substrate, we obtain an enzyme system in which, on balance, the molecular oxygen oxidizes the specific substrate, e.g.

$O_2 \rightarrow$ cuprous $\rightarrow$ cupric $\rightarrow$ catechol $\rightarrow$ *o*-quinone

$\rightarrow$ dihydropyridine $\rightarrow$ pyridine $\rightarrow$ Robison ester / alcohol / lactic acid / etc.

Such combined enzyme systems are 'oxidases' which are inhibited by cyanide and carbon monoxide, but the carbon monoxide inhibition is not, however, light sensitive.

8. Relationship with the haemocyanins

If the oxygen transporting blood pigments have developed from the oxygen transporting enzymes, then the haemocyanins have been evolved from the oxygen transporting copper proteids and should therefore be related chemically. A comparison would support such a conception.

According to Hernler and Philippi† the haemocyanins have the following copper contents:

Haemocyanin from Helix pomatia . . .	0·245%
Haemocyanin from Loligo pealei (Squid) . .	0·26%
Haemocyanin from Limulus polyphemus . .	0·173%
Haemocyanin from Homarus americanus . .	0·187%
Haemocyanin from Dramia vulgaris . . .	0·17%

The copper content of phenol oxidase (0·2 per cent.) lies within these values.

Further confirmation is met with in the ratio carbon mon-

† F. Hernler and E. Philippi, *Zeitschr. f. physiologische Chem.* **216**, 110 (1933).

oxide:copper. R. W. Root† found for the carbon monoxide haemocyanins the ratio CO:Cu — 1:2. We found the same ratio for the carbon monoxide compound of phenol oxidase.‡ It should be noted that this ratio is not perhaps generally applicable for the carbon monoxide compounds of copper. In the carbon monoxide compound of cuprous chloride, for example, the ratio CO:Cu = 1:1.

The most essential point of confirmation, however, is that the method of splitting and resynthesizing the enzyme can be applied with the same success to the haemocyanins. The haemocyanins are split up by dialysis against hydrogen cyanide into copper salt and protein and they can be resynthesized by uniting the two parts. From this similarity in behaviour it must be concluded that the copper in the haemocyanins is combined in the same way as in the enzyme. Nevertheless the two classes of substances, in accordance with their function, show a different behaviour towards oxygen. The monovalent copper of the haemocyanins takes up molecular oxygen but is not oxidized by it to divalent copper, whilst the monovalent copper of the enzyme is oxidized. The absorption spectra of the two substances, on saturating their solutions with oxygen, are therefore different. The oxygen compounds of the univalent copper of the haemocyanins are blue and have characteristic absorption bands. The cupric derivatives of the enzyme are yellowish in colour and have no characteristic bands.

9. Other oxygen transporting copper proteids

'Polyphenol oxidase, purification, nature and properties' is the title of a later publication by Keilin and Mann.§ In this publication, they proved that phenol oxidase is a copper proteid. Instead of potatoes they used mushrooms as the starting material. Their statement that the mushroom oxidase is 300 times more active than that of the potato was due to an error in calculation. Both copper proteids have about the same activity.

† R. W. Root, *Journ. biolog. Chem.* **104,** 239 (1934).
‡ F. Kubowitz, *Bioch. Zeitschr.* **299,** 32 (1938).
§ D. Keilin and T. Mann, *Proc. Royal Soc. London,* B **125,** 187 (1938).

The phenol oxidase from the latex of *Rhus succedanea* is also a copper proteid as Keilin and Mann† showed in 1939. Bertrand named this oxidase, laccase in 1894. This copper proteid is different from the phenol oxidase of the potato and the mushroom in having a blue colour and in being uninhibited by carbon monoxide. It is probable that the blue laccase is a non-dissociating compound of oxygen with the monovalent copper present.

Lovett-Janison and Nelson‡ isolated another oxygen transporting copper proteid in 1940 from pumpkin seed. It contains 0·15 per cent. copper and is a specific ascorbic acid oxidase.

If I am correct in my opinion that the haemocyanins have been evolved from oxygen transporting enzymes, then animal cells should also have enzymes which are capable of transporting oxygen by means of copper. In the future a look-out should be kept for animal cells the carbon monoxide inhibited respiration of which is only partly light sensitive. A respiration partly non-light sensitive would indicate that copper proteids are involved.

† D. Keilin and T. Mann, *Nature*, **143**, 23 (1939).

‡ P. L. Lovett-Janison and J. M. Nelson, *Journ. Amer. Chem. Soc.* **62**, 1409 (1940).

CHAPTER XVIII

THE ABSORPTION SPECTRUM OF A FERMENTING ENZYME EVOLVING HYDROGEN

SINCE butyric acid fermentation†

$$C_6H_{12}O_6 = C_4H_8O_2 + 2CO_2 + 2H_2$$

is, as W. Kempner found, inhibited by carbon monoxide, and since this inhibition is light sensitive, butyric acid bacteria must contain an enzyme which reacts reversibly with carbon monoxide and has a reacting group containing iron

$$Fe + CO \rightleftharpoons FeCO.$$

We have determined the absorption spectrum of this enzyme—'wasserstoffentwickelnden Gärungsferment'—using the photochemical method. Surviving butyric acid bacteria, the fermenting action of which had been inhibited by carbon monoxide, were exposed to monochromatic light of known intensity. The spectrum of the carbon monoxide compound was determined from the increase in fermentation on exposure. Any confusion with the respiration inhibiting action of the carbon monoxide was excluded since the anaerobically cultured bacteria have no oxygen transporting iron, and, moreover, all fermentation determinations were made under conditions of oxygen exclusion.

The mathematical theory is very simple in this case on account of the conditions being anaerobic. The model is the formation and dissociation of the carbon monoxide haemochromogen the equations for which have previously been worked out.‡ Technically, however, there were difficulties in obtaining the intensities of monochromatic light necessary for the decomposition of the carbon monoxide compound of the enzyme because the carbon monoxide inhibition of butyric acid fermentation is a hundred times less sensitive to light than the carbon monoxide inhibition of respiration.

† Section 12, Chapter IX. W. Kempner, *Bioch. Zeitschr.* **257**, 41 (1933); W. Kempner and F. Kubowitz, ibid. **265**, 245 (1933); F. Kubowitz, ibid. **274**, 285 (1934).

‡ Section 5, Chapter XI.

1. Dark equilibrium

When the butyric acid fermentation is determined at different partial pressures of carbon monoxide it is found that the quantity

$$\frac{\text{residual fermentation}}{\text{inhibited fermentation}} \cdot \text{carbon monoxide pressure}$$

is constant. Let Fe be the iron of the enzyme reacting with carbon monoxide and p_{CO} the partial pressure of carbon monoxide, then at equilibrium

$$\frac{\text{Fe}\, p_{CO}}{\text{FeCO}} = K.$$

The equilibrium constant K depends on the H ion concentration and the temperature. At 8° and pH 6·6 it has the value $1{\cdot}5 \times 10^{-2}$ [atmos.].

Therefore at a carbon monoxide pressure of 0·015 atmos., half of the iron of the enzyme is combined with carbon monoxide and the butyric fermentation is therefore half inhibited.

2. Light sensitivity†

When butyric acid bacteria, the fermenting action of which has been inhibited by carbon monoxide, are exposed to light the equilibrium constant K is increased from the dark value K_d to the light value K_h which is determined by the intensity and the wave-length of the light. The question as to how this difference between the two equilibrium constants, $K_h - K_d = \Delta K$, is dependent on the light intensity is answered in the following series of experiments carried out at 8° and pH 6·6. White light from a carbon arc which had the ultra-violet and heat radiation filtered out was used.

Light intensity i' $\left[\frac{\text{cal.}}{\text{cm.}^2 \text{ minutes}}\right]$	*Equilibrium Constant* K [Atmos.]	$\frac{\Delta K}{K_d}$ [0]	$\frac{1}{i'}\frac{\Delta K}{K_d}$ $\left[\frac{\text{cm.}^2 \text{ minutes}}{\text{cal.}}\right]$
0	$K_d = 1{\cdot}55 \times 10^{-2}$	..	..
$2{\cdot}79 \times 10^{-2}$	$K_h = 2{\cdot}37 \times 10^{-2}$	0·532	19
$6{\cdot}73 \times 10^{-2}$	$K_h = 3{\cdot}60 \times 10^{-2}$	1·33	20
$17{\cdot}20 \times 10^{-2}$	$K_h = 7{\cdot}18 \times 10^{-2}$	3·64	21

† Section 1, Chapter XIII.

When, therefore, the light intensity i' was varied within a factor of 6, $\frac{\Delta K}{i'}$ only changed about 10 per cent. This means that ΔK was very nearly proportional to the light intensity.

On the basis of this result we can express L, the light sensitivity of the carbon monoxide compound of the enzyme as

$$L = \frac{1}{i}\frac{\Delta K}{K_d}$$

where i is the quantum intensity of the light.

At 8°, for wave-lengths 436 mμ and 546 mμ we obtained the following values:

$$L_{436} = 2{\cdot}6 \times 10^6 \left[\frac{\text{cm.}^2 \text{ minutes}}{\text{mole quanta}}\right]$$

$$L_{546} = 0{\cdot}88 \times 10^6 \left[\frac{\text{cm.}^2 \text{ minutes}}{\text{mole quanta}}\right],$$

whilst at 10° the light sensitivity of the carbon monoxide compound of the oxygen transporting enzyme of yeast amounted to:

$$L_{436} = 7{\cdot}6 \times 10^8 \left[\frac{\text{cm.}^2 \text{ minutes}}{\text{mole quanta}}\right]$$

$$L_{546} = 0{\cdot}73 \times 10^8 \left[\frac{\text{cm.}^2 \text{ minutes}}{\text{mole quanta}}\right].$$

At 546 mμ the oxygen transporting enzyme is therefore 83 times, and at 436 mμ, 290 times more sensitive than the hydrogen evolving enzyme of butyric acid bacteria, the temperature in both cases being about the same.

3. Relative absorption spectrum

If butyric acid bacteria, the respiration of which is inhibited by carbon monoxide, are exposed to light of wave-lengths λ_1 and λ_2, and the corresponding light sensitivities are L_1 and L_2, then by applying equation (10) of Chapter XIII we obtain

$$L_1 = \frac{\phi_1 \beta_1}{z_d},$$

$$L_2 = \frac{\phi_2 \beta_2}{z_d},$$

and if we assume that the photochemical yield is the same for all regions of the spectrum, i.e. $\phi_1 = \phi_2$ then:

$$\frac{I_1}{I_2} = \frac{\beta_1}{\beta_2}. \tag{1}$$

Using this equation Kempner and Kubowitz determined in 1933 the relative absorption spectrum of the carbon monoxide compound of the enzyme. Later on, Kubowitz, using a stronger mercury lamp (220 volt, 22 amp., without a vacuum) obtained the following rather more accurate values:

Light source	*Wave-length* [mμ]	$\frac{\beta_\lambda}{\beta_{436}}$
Mercury lamp	366	2·6
,,	385	1·95
,,	405	1·70
,,	436	1·00
,,	495	0·606
,,	546	0·27
,,	578	0·19
Carbon arc	650	0·038

The enzyme therefore showed absorption over the whole of the spectrum examined, the absorption being greater the shorter the wave-length. The spectrum appears as though it were leading up to a band in the ultra-violet, although it may be that the number of wave-lengths used was inadequate.

4. Dark dissociation constant

When butyric acid bacteria, the respiration of which has been inhibited by carbon monoxide, are exposed to light and then the light is cut off, the dark dissociation constant of the hydrogen forming enzyme can be determined from the rate at which the dark equilibrium is attained from the light equilibrium. For the calculation equation (38) of Chapter XI is used, the uninhibited respiration A_0 being merely replaced by the uninhibited fermentation G_0,

light to dark:

$$G_0 \int_0^t n\,dt = G_0 n_d t + G_0 \frac{n_h - n_d}{\omega_d + \rho}\left(1 - e^{-(\omega_d+\rho)t}\right). \tag{2}$$

On exposure to strong white light of undetermined intensity at 8° and at pH 6·65 in M/20 phosphate containing 1 per cent. glucose we found $G_0 = 2{\cdot}35$ mm. per minute; n_d 0·377; n_h 0·797. On darkening and determining the fermentation at intervals of 2 min., the summations $G_0 \int_0^t n\,dt$ were obtained. Thus all the quantities in equation (2) were known with the exception of $(\omega_d+\rho)$ which was then obtained by test. We found:

t after darkening [minutes]	$G_0 \int_0^t n\,dt$ [mm.]	$(\omega_d+\rho)$ $\left[\frac{1}{\text{minutes}}\right]$
2	3·14	0·40
4	5·63	0·40
6	7·73	0·38
8	9·73	0·35
10	11·61	0·35
		Mean 0·38

If we multiply this value for $(\omega_d+\rho)$ with n_d we obtain in accordance with equation (12) of Chapter XI the dark dissociation constant:

$$z_d = \omega_d = n_d(\omega_d+\rho) = 0{\cdot}377\times 0{\cdot}38 = 0{\cdot}143\left[\frac{1}{\text{min.}}\right].$$

$z_d = 0{\cdot}143$ signifies that in the dark at 8°, 14·3 per cent. of the FeCO compound present dissociates per minute into Fe and CO. z_d at 10° for the oxygen transporting enzyme of yeast was about three times greater. We therefore had to expose and darken several times in the z_d determination of the yeast enzyme, whilst in this case the reversal to the dark equilibrium proceeded so slowly that a single darkening was sufficient for the determination. The photochemical determination of the spectrum of the fermentation enzyme was easier, not only theoretically but also experimentally, than that of the oxygen transporting enzyme.

5. Molar absorption spectrum

Butyric acid bacteria, the fermenting action of which had been inhibited by carbon monoxide were exposed at 8° to light of measured intensity and wave-length 436 mμ. The fermentation thereby increased from $n_d = 0{\cdot}337$ to $n_h = 0{\cdot}453$. The light

intensity i producing this light value amounted to

$$3{\cdot}18\times10^{-7}\left[\frac{\text{mole quanta}}{\text{cm.}^2\text{ minutes}}\right].$$

From these data we obtained the light sensitivity L for wave-length 436 mμ and at a temperature of 8°:

$$L_{436}=\frac{1}{i}\frac{\Delta K}{K_d}=2\times10^6\left[\frac{\text{cm.}^2\text{ minutes}}{\text{mole quanta}}\right].$$

The dark dissociation constant z_d determined for the same bacterial suspension and under the same external conditions was found to be 0·143.

On applying equation (10), Chapter XIII,

$$L_{436}=\frac{\phi\beta_{436}}{z_d},$$

and, with $\phi=1$,

$$\beta_{436}=z_d\frac{L_{436}}{\phi}=0{\cdot}143\,\frac{2\times10^6}{1}=2{\cdot}86\times10^5\left[\frac{\text{cm.}^2}{\text{gram atoms Fe}}\right].$$

Using this value for β to convert the relative into the molar absorption spectrum we obtain:

Wave-length [mμ]	β $\left[\frac{\text{cm.}^2}{\text{gram atoms iron}}\right]$
366	$7{\cdot}4\times10^5$
385	$5{\cdot}6\times10^5$
405	$4{\cdot}9\times10^5$
436	$2{\cdot}86\times10^5$
495	$1{\cdot}7\times10^5$
546	$0{\cdot}77\times10^5$
578	$0{\cdot}54\times10^5$
650	$0{\cdot}11\times10^5$

These figures are used to plot the absorption spectrum in Fig. 28.

6. The chemical combination of the iron

From the shape of the absorption spectrum and also from the magnitude of the molar absorption coefficients it follows that the hydrogen forming fermentation enzyme cannot be a haem compound. The carbon monoxide haemochromogens have their main absorption bands in the blue region, and the molar light

absorption coefficients are of the order of 10^8, whilst in the case of the hydrogen forming fermentation enzyme we found values of the order of 10^5.

In so far as the magnitude of the light absorption coefficients is concerned, the SH-complexes of iron are more closely related

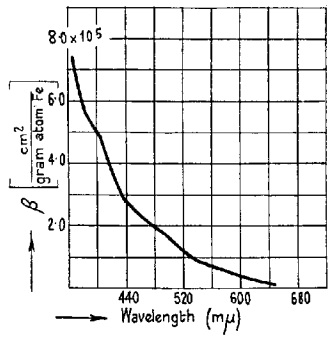


FIG. 28. Molar absorption spectrum of the carbon monoxide compound of the hydrogen evolving fermentation enzyme calculated for $\phi = 1$.

to the fermentation enzyme. For example, for these compounds we found:†

Carbon monoxide compound of		ferrous cysteine	$= 5{\cdot}0 \times 10^5$
,,	,,	ferrous thiolactic acid	$= 7{\cdot}6 \times 10^5$
,,	,,	ferrous glutathione	$= 14{\cdot}0 \times 10^5$.

In spite of this, however, the enzyme cannot be an iron-sulphur compound, since the iron in these sulphur compounds reacts with two molecules of carbon monoxide. In their equilibrium constant the square of the carbon monoxide pressure is involved,

$$\frac{\mathrm{Fe}(p_{\mathrm{CO}})^2}{\mathrm{Fe(CO)_2}} = K,$$

whilst in the equilibrium constant for the carbon monoxide compound of the enzyme the carbon monoxide pressure has the index 1‡.

$$\frac{\mathrm{Fe}\, p_{\mathrm{CO}}}{\mathrm{FeCO}} = K.$$

† W. Cremer, *Bioch. Zeitschr.* **206**, 228 (1929); F. Kubowitz, ibid. **282**, 277 (1935).

‡ Section 1 of this chapter.

7. Mechanism of the action of iron

As with the nature of combination of the iron, the reaction mechanism of the iron in the hydrogen forming fermentation enzyme is also unknown. Till recently there seemed to be little doubt that the iron reacted by virtue of a valency change. Since, however, a heavy metal catalysis has been discovered in yeast fermentation† which is not dependent on a valency change of the heavy metal, it is not now possible to express any view on the reaction mechanism of the iron in the hydrogen forming enzyme.

Finally it should be mentioned that among the Bredig enzyme models‡ there is one for the hydrogen forming fermentation enzyme, namely, finely divided rhodium, which decomposes formic acid according to the equation

$$HCOOH = H_2 + CO_2.$$

† Chapter XIX.

‡ Th. Blackadder, *Zeitschr. f. physikalische Chemie*, **81**, 385 (1913).

CHAPTER XIX

HEAVY METALS AND YEAST FERMENTATION

N/100 hydrogen cyanide inhibits fermentation by living yeast, whilst the narcotic inhibition appears only when the concentration of the cyanide is about twice normal. Heavy metals therefore, as was recognized in 1925,† are concerned in yeast fermentation. Only the separation and isolation of the fermentation enzyme, however, has made it possible to investigate which part of the fermentation involves the action of heavy metals.

Enolase and phosphate transporting enzymes, which are activated by magnesium salts, can also be activated by zinc and manganese salts. If, however, zinc and manganese salts in the yeast cells are combined with substances forming complexes with heavy metals, enolase and phosphate transporting enzymes are not inhibited since yeast cells contain more than sufficient magnesium salts.

There is, however, a fermentation enzyme, zymohexase, which is activated by heavy metal salts but not by magnesium salts. Zymohexase splits hexose diphosphate into triose phosphate. If the heavy metal salts of yeast cells are combined with substances forming heavy metal complexes the zymohexase should be inhibited. This being so it seems likely that it is the inactivation of zymohexase which is involved in the inhibition of yeast fermentation by substances forming complexes with heavy metals.

1. Method

When zymohexase splits hexose diphosphate into triose phosphate the equation for the reaction is:‡

1 hexose diphosphate

$$\rightleftharpoons 1 \text{ aldotriose phosphate} + 1 \text{ ketotriose phosphate.} \quad (1)$$

† O. Warburg, *Katalytische Wirkungen der lebendigen Substanz*, Berlin, 1928 (p. 11); *Bioch. Zeitschr.* **165**, 196 (1925); Zuckerkandl and Messimer-Klebermann, ibid. **261**, 55 (1933).

‡ O. Meyerhof and K. Lohmann, ibid. **271**, 102 (1934).

If the solution in which this reaction takes place contains, in addition to the oxidizing fermentation enzyme, pyridine nucleotide and arsenic acid, the reversible reaction (1) is followed by the irreversible reaction:†

1 aldotriose phosphate + 1 pyridine nucleotide + 1 H_2O
= 1 phosphoglyceric acid + 1 dihydropyridine nucleotide. (2)

Since of all the components in reactions (1) and (2), only the

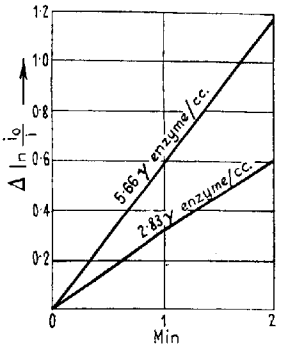


FIG. 29. Optical zymohexase test. i_0/i is the light absorption for wave-length 340 mμ and layer thickness 0·574 cm.

dihydropyridine nucleotide absorbs in the long-wave ultra-violet region, a light absorption takes place in this region as hexose diphosphate is decomposed. This can be measured photoelectrically. The facts described below were discovered by means of this 'optical test'.†

2. Behaviour of zymohexase towards complex-forming substances and heavy metal salts

Yeast zymohexase is inhibited by complex-forming substances of different chemical composition, for example, by cysteine, α-α′-dipyridyl, and pyrophosphate. The addition of salts of zinc, iron, or cobalt, prevents the inhibition or abolishes

† O. Warburg and W. Christian, ibid. **311**, 209 (1942); **314**, 149, 399 (1943).

it if it has already taken place. According to a principle† to which there have been no exceptions since 1923, it follows that yeast zymohexase is a dissociating heavy metal compound.

There is no contradiction of this view in that yeast zymohexase usually reacts even although heavy metal salts have not been added to the test solutions. This is because the small amounts of heavy metal which are present as impurities are always more than sufficient to saturate the enzyme protein with metal. If the equivalent weight of zymohexase is 100,000 g. and 1 c.c. of the test solution contains 10 γ enzyme protein, 0·006 γ of zinc or iron is sufficient to activate the protein of zymohexase in 1 c.c. Wilhelm Lüttgens found in 1 c.c. of our test solution 0·15 γ zinc and 0·35 γ iron as impurity.

If it is desirable under these conditions to remove heavy metal salts from a test solution so that the enzyme dissociates into protein and metal salt, the only method available is to add a complex-forming substance. When the enzyme is inactivated in this way it can be reactivated by the addition of metal salts in amounts great in comparison with the enzyme protein, but small in comparison with the amount of complex forming substance.

3. Cysteine and zinc salts

In our test solutions M/50 cysteine inhibited the zymohexase very strongly at neutral reaction. The activity of the enzyme was restored partly on adding M/50,000 zinc sulphate and completely by M/5,000 zinc sulphate (Fig. 30).

In view of what follows it is to be emphasized that the activation by zinc salts is independent of the oxygen pressure. If the added zinc salt is sufficient to convert the total enzyme protein into zinc zymohexase, the enzyme action is the same whether the test solution is saturated with argon or with oxygen.

4. Cysteine and iron salts

As with zinc sulphate the zymohexase inhibited by cysteine

† O. Warburg and S. Sakuma, *Pflügers Archiv*, **200**, 203 (1923); S. Sakuma, *Bioch. Zeitschr.* **142**, 68 (1923). For the applications of our principle, cf. for example J. Beyer and M. J. Johnson, *Journ. biolog. Chem.* **130**, 641, 655 (1939); E. Maschmann, *Ergebnisse*, **9**, 155 (1943).

can be reactivated by iron salts. This activation is, however, dependent on the oxygen pressure. The greater the oxygen concentration the smaller is the activation by iron salts. The extent of the activation is greatest in the absence of oxygen, and is almost zero with oxygen at atmospheric pressure. If the enzyme is inhibited by cysteine and activated by iron salts

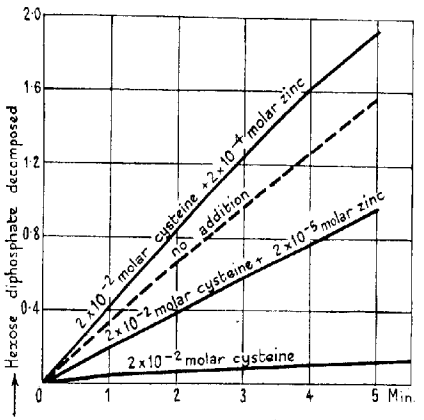


FIG. 30. Inhibition of yeast zymohexase and activation by zinc sulphate.

it is possible by changing over from anaerobic to aerobic conditions to cause the activity of the zymohexase to disappear (Fig. 31).

It follows from this that it is the divalent and not the trivalent iron which activates the zymohexase. In a cysteine solution which is free from oxygen the iron is present in the ferrous state, whilst on saturation with oxygen at atmospheric pressure, almost all the iron is present in the ferric state. Also the reactions brought about by both the valency changes, i.e. the reduction of the ferric cysteine by excess of cysteine and the oxidation of the ferrous cysteine by molecular oxygen, are fast.

The suspicion naturally arises that the key to the solution of the Pasteur reaction is to be found here. The Pasteur reaction

may be nothing other than the oxidation of a complex ferrous compound in the fermenting cell. However, it is necessary to postpone consideration of this theory till it has been explained why animal zymohexase behaves differently from yeast zymohexase.

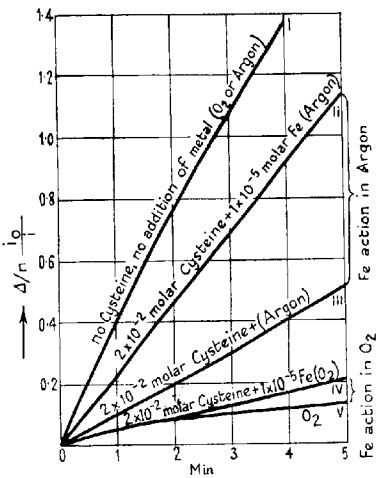


FIG. 31. Inhibition of yeast zymohexase by cysteine and reactivation by iron salts. Influence of oxygen on the reactivation.

5. Cysteine and cobalt salts

Zymohexase inhibited by cysteine can also be activated by cobalt sulphate. It is necessary, however, to remove the oxygen from the solutions before adding the cobalt. Cobaltous cysteine is oxidized rapidly by oxygen to cobaltic cysteine, whilst cobaltic cysteine in contrast to ferric cysteine, is only slowly reduced by excess cysteine to the cobaltous salt. When, therefore, the divalent cobalt has been oxidized to the trivalent state it takes a long time after exclusion of the oxygen till the divalent cobalt is formed and the action of the zymohexase again begins. Thus iron zymohexase and cobalt zymohexase differ considerably from one another. On changing from aerobic

to anaerobic conditions only iron zymohexase reacts as quickly as fermentation takes place in the cell.

6. α-α′-Dipyridyl

α-α′-Dipyridyl and *o*-phenanthroline combine with heavy metal salts to give complex compounds. The best known of these are the ferrous complexes, distinguished by their red colours. They are more stable in acid than in neutral solution, and although ferrous compounds, they are not autoxidizable.

We found that yeast zymohexase was inhibited by α-α′-dipyridyl. As was expected from the above, the inhibition was greater in acid than in neutral solution, and the oxygen pressure had no effect on the inhibition.

For example, at 20° we found:

pH 7·4

$[Dipyridyl]$ $\left[\frac{\text{mole}}{\text{litre}}\right]$	*Inhibition* %	$\frac{\textit{Residual activity}}{\textit{Inhibition}} \times [Dipyridyl]^2$ $\left[\left(\frac{\text{mole}}{\text{litre}}\right)^2\right]$
$1{\cdot}07\times10^{-3}$	..	..
$3{\cdot}2\ \times10^{-3}$	38	$16{\cdot}7\times10^{-6}$
$10{\cdot}7\ \times10^{-3}$	89	$14{\cdot}4\times10^{-6}$

pH 6·4

$[Dipyridyl]$ $\left[\frac{\text{mole}}{\text{litre}}\right]$	*Inhibition* %	$\frac{\textit{Residual activity}}{\textit{Inhibition}} \times [Dipyridyl]^2$ $\left[\left(\frac{\text{mole}}{\text{litre}}\right)^2\right]$
$0{\cdot}3\times10^{-3}$	25	$0{\cdot}27\times10^{-6}$
$1{\cdot}0\times10^{-3}$	77	$0{\cdot}30\times10^{-6}$
$3{\cdot}0\times10^{-3}$	94	$(0{\cdot}58\times10^{-6})$

From the table it can be seen that the expression

$$\frac{\text{residual activity}}{\text{inhibited activity}} \times [\text{dipyridyl}]^2$$

is almost constant at a given pH, and is greater at pH 7·4 than at pH 6·4. The dipyridyl concentrations producing half

inhibition of the zymohexase were:

$$\text{at pH 7·4: } \sqrt{(15\times10^{-6})} = 3{\cdot}9\times10^{-3}\,\frac{\text{mole}}{\text{litre}},$$

$$\text{at pH 6·4: } \sqrt{(0{\cdot}28\times10^{-6})} = 0{\cdot}53\times10^{-3}\,\frac{\text{mole}}{\text{litre}},$$

i.e. at pH 6·4 dipyridyl is seven times more active than at

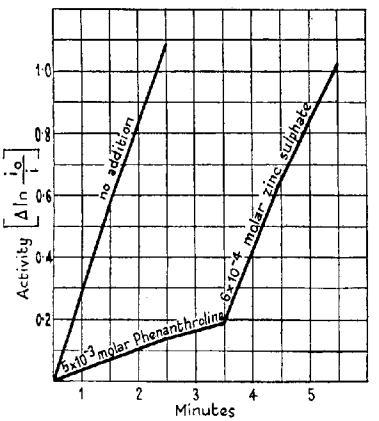


FIG. 32. Inhibition of yeast zymohexase by phenanthroline and reactivation by zinc sulphate. pH 7·4. 20°.

pH 7·4. *o*-Phenanthroline behaves similarly to dipyridyl in inhibiting yeast zymohexase.

When zymohexase had been inhibited by dipyridyl or phenanthroline, the activity could be restored by zinc sulphate (Fig. 32).

7. Pyrophosphate

Yeast zymohexase is inhibited by pyrophosphate. For example, at pH 7·4 and 20°:

	In the 1st minute after adding enzyme	*In the 5th minute after adding enzyme*
	Inhibition	*Inhibition*
In M/1,000 pyrophosphate	0	22%
„ M/300 „	21%	67%
„ M/100 „	58%	88%

The inhibition thus increased with time as is characteristic of irreversible inhibitions.

That the inhibition is really associated with irreversible changes is proved by the behaviour of zinc sulphate. When a small amount of zinc sulphate was added to the pyrophosphate before introduction of the enzyme, there was no inhibition. When, however, the zinc sulphate was added after the pyrophosphate inhibition was complete, i.e. after about five minutes, there was no reversal of effect.

8. The mechanism of the action of the metal salts

The most that can be said about this is that the action of the metal salts is different from that taking place in the oxygen transport by the iron and copper of oxygen transporting enzymes. The mechanism of such oxygen transport involves the valency change of the heavy metal. Since, however, zinc, which cannot change its valency, is included amongst those metals which activate zymohexase, a valency change mechanism is excluded for zymohexase even in the case of iron and cobalt salts.

A further fundamental difference is the low specificity as regards the heavy metal. There are at least three heavy metals the salts of which activate zymohexase; these are zinc, iron, and cobalt. In the case of the oxygen transporting enzymes, no metal can replace any other.

9. Muscle zymohexase

Muscle zymohexase which we obtained crystalline from rat muscle, is not inhibited by substances forming complexes with heavy metals. Neither cysteine, nor α-α'-dipyridyl, nor pyrophosphate has any inhibitory effect, and the uninhibited action of muscle zymohexase in solutions containing complex-forming substances is not increased by addition of metal salts. The zymohexase isolated from yeast cells and that from muscle therefore behave towards complex-forming substances, at any rate *in vitro*, in quite a different manner. (Cf. the work of Uvstedt†

† H. J. Uvstedt, *Bioch. Zeitschr.* **265**, 154 (1933).

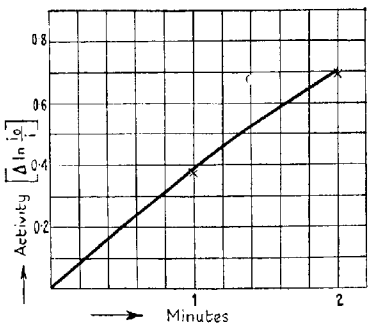


FIG. 33. Non-inhibition of muscle zymohexase by dipyridyl (20°, pH 6·4). Line: no dipyridyl. Crosses: 10^{-2} M dipyridyl.

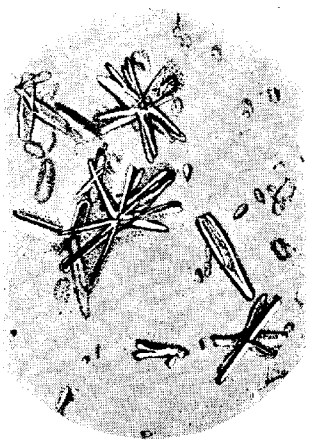


FIG. 34. Muscle zymohexase from ammonium sulphate.

and von Zuckerkandl† on the inhibition of the total fermentation by phenanthroline.

This discovery that two enzymes possessing the same chemical action behave so differently towards complex-forming sub-

† F. Zuckerkandl, W. Fleischmann, and G. Drucker, *Bioch. Zeitschr.* **271**, 435 (1934).

stances appeared so remarkable that we thought it worth while to see if the effect was due to the presence of substances, or even enzymes, closely associated with the zymohexase. If this were so, the difference in the behaviour would disappear when the purified zymohexases were used. But the muscle zymohexase, whether pure or in crude muscle extracts, was uninhibited by complex-forming substances (Fig. 33), and the yeast zymohexase, both in the pure state and in crude extracts, remained inhibited. In mixtures of the two purified zymohexases, the muscle zymohexase was uninhibited, whilst yeast zymohexase in the mixture was inhibited to exactly the same degree as in the absence of the muscle enzyme.

Further, chemical analysis of the two zymohexases (only the muscle substance has as yet been obtained crystalline (Fig. 34)) revealed no difference. Taking everything into consideration, I feel as Mitscherlich did one hundred years ago when he was puzzled over the action of hydrogen cyanide:† I think the phenomenon is very puzzling and I attach much importance to the explanation.

† Cf. section 1, Chapter III.

CHAPTER XX

PHOTOCHEMICAL REDUCTION OF QUINONE IN GREEN CELLS AND GRANULES

This work, carried out in collaboration with Wilhelm Lüttgens in 1944–5, has been published in Berichte der Moskauer Academie der Wissenschaften.

WHEN dried green leaves are ground up with water and exposed to light, oxygen is evolved, as Molisch† found in 1925 using the 'Leuchtbakterien' method. The experiment has been repeated and verified frequently. No loss of carbon dioxide is observed. The oxygen must therefore be produced from a substance other than carbon dioxide.

In 1939 Robert Hill‡ replaced the method employing bacteria by a quantitative method. He added haemoglobin to the leaf material, and when the oxygen was produced on exposure to light, oxyhaemoglobin was formed and the amount could be determined by an optical method. Using this technique, Hill discovered that isolated chloroplasts on exposure to light reduced certain ferric salts, in particular, ferric oxalate, with evolution of oxygen. Now it was known that ferric oxalate could be reduced in light to ferrous oxalate and carbon dioxide. The latter might then be responsible for the oxygen. But Hill was able to exclude such a mechanism.

The experiments described in the following may be considered as an extension of those of Molisch and Hill. They were undertaken with a view to separating the photochemically active system of green cells from their structure. This has been successful in as far as we found that neither the cell structure nor the chloroplast structure were necessary for the photochemical formation of oxygen. But we have not been able to obtain the photochemically active system in solution. The photo-reaction is, however, still susceptible to narcotics as *in vivo*, and is therefore a surface reaction.

† Molisch, *Zeitschrift für Botanik*, **17**, 577 (1925).
‡ Robert Hill, *Proc. Royal Soc.* B **127**, 192 (1939).

1. Discovery of the quinone reduction†

When a suspension of surviving Chlorella is shaken in a manometer vessel containing potash, a negative pressure is developed owing to the respiration of oxygen. On exposure to light, the reduction in pressure becomes less on account of the evolution of oxygen from the respiratory carbon dioxide, and under sufficiently strong illumination the reduction in pressure becomes zero. This is not of course a method of determining the photochemical yield, but it is a simple and sensitive method for showing whether oxygen is developed on exposure to light.

If, in the same way, respiring press juice from spinach leaves or sugar beet leaves is exposed to light and then darkened the pressure reductions are the same both in the light and in the dark. This means that in the press juices exposed to light no oxygen is formed from the respiratory carbon dioxide—a result which has always surprised us and which we have verified many times.

We were, moreover, disturbed to find that the oxygen uptake of the press juice in the dark decreased with time, and at an irregular rate. With a view to preventing this decrease we added several substances, among others, catechol and hydroquinone. Actually these substances stabilized the oxygen uptake in the dark by being oxidized in the press juice to quinones. On exposing the mixture to light it was seen that a photo-reaction was taking place. The oxygen requirement became smaller in the light and even changed over with sufficiently strong illumination to a production of oxygen.

We thus discovered that on illumination quinones are reduced by green press juice with oxygen evolution according to the equation

$$2\ \text{quinone} + 2H_2O = 2\ \text{hydroquinone} + O_2.$$

Naphthoquinones, for example, naphthoquinone sulphonic acid, are likewise reduced with evolution of oxygen. Unfortunately we have not been able to try naturally occurring naphthoquinones such as 2-methyl-3-phytyl-1:4-naphthoquinone (vitamin K_1).

† Private communication, cf. O. Warburg and W. Lüttgens, *Die Naturwissenschaften*, April–June and October, 1944.

2. Oxygen requirement of green leaf extract

Spinach leaves or sugar beet leaves were put through a mincing machine and then pressed through cloth. By centrifuging for a short time at a speed corresponding to 1,200 times gravity, cells and undissolved salts were removed from the press juice. The dark-green liquid containing a suspension of intact chloroplasts and granules derived from them is referred to in the following as 'green extract'. The pH of the extract was about 6·5. When the green extract was shaken with air, the oxygen uptake decreased with time and was inhibited by a mixture of 80 per cent. carbon monoxide and 20 per cent. oxygen by volume to the extent of 50 per cent. Light had no effect on the inhibition. In view of this inhibition by carbon monoxide and its insensitivity to light, it can be concluded that oxygen is being transported by the copper† of a phenol oxidase in the extract. This is in agreement with the fact that the oxygen uptake is increased by catechol. The oxygen requirement, even when increased by the addition of catechol, still decreased with time. In order to prevent this decrease we added with the catechol a large amount of hydroquinone which is not oxidized by the phenol oxidase, and which by itself did not increase the oxygen uptake. With hydroquinone and catechol the reaction is:

$$2Cu^{++} + \text{catechol} = 2Cu^{+} + \text{orthoquinone} + 2H^{+}$$

$$\text{orthoquinone} + \text{hydroquinone} = \text{catechol} + p\text{-quinone},$$

so that the catechol is no longer the only catalyst, and the end product of the oxidation, instead of being poisonous *o*-quinone and its oxidation products, is the less-poisonous hydroquinone.

Actually if relatively large amounts of hydroquinone and a little catechol are added to the green extracts there is obtained an oxygen uptake which remains constant until all the hydroquinone has been oxidized to quinone.

In the three experiments which are illustrated in Fig. 35, the oxygen uptake and the carbon dioxide formation were determined and found to be:

† O. Warburg, *Ergebnisse der Enzymforschung*, **7**, 210 (1938).

no addition . . . . . $CO_2/O_2 = 0{\cdot}7$ to $1{\cdot}0$

after addition of catechol . . . „ $= 0{\cdot}3$ to $0{\cdot}4$

after addition of catechol + hydroquinone „ $= 0$.

This means that the oxygen used or transported by the catechol gives rise to no carbon dioxide. Hydroquinone inhibits the original carbon dioxide formation and therefore apparently displaces the natural substrate of the respiration.

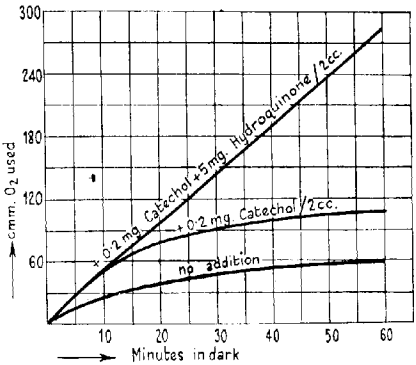


FIG. 35. Oxygen requirement of green leaf extract in the dark. 2 c.c. extract. 20°. pH 6·5. Gas space, air; potash in vessel.

Both the suspended matter and the liquid in the green extract take up oxygen. When these are separated by centrifuging at pH 6·5, the pH of the extract, and catechol and hydroquinone are added to both fractions, about a quarter of the oxygen requirement is found in the sediment and three-quarters in the liquid. If centrifuging is carried out at stronger acid reaction, for example, at pH 5, a large fraction of the phenol oxidase sediments. The sediment then contains, in addition to the phenol oxidase of the suspended matter, some also which has been precipitated by the acid from the liquid. If it is required therefore, as in the following photochemical experiments, to separate the phenol oxidase of the suspended matter from that of the liquid, the green extract must be centrifuged at pH 6·5.

3. Action of light on the oxygen uptake of green leaf extract

In order to test whether the oxygen uptake of green leaf extract is sensitive to light we shook in air the same amount of an extract in a darkened and in an illuminated vessel side by side. In other experiments we used only one vessel, and alternately darkened it and exposed it to light. The second method was preferable, but requires the supposition that the oxygen uptake in the dark remains constant with time.

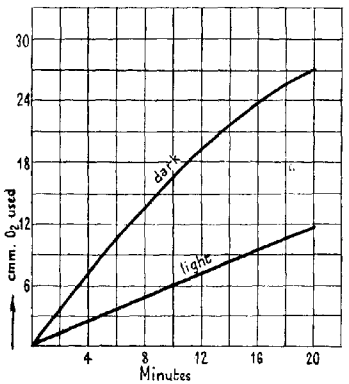


FIG. 36. Action of light on the catechol oxidation in the green sediments. 0·2 mg. catechol, 2 cm.³ suspension. 20°. pH 6·5. Air in gas space.

A low-voltage metal filament lamp (11 volt, 38 amp.) served as light source, from which, with a condenser and lenses, a parallel beam was obtained. This entered the thermostat, after filtration through 2 c.c. of 20 per cent. acid ferrous sulphate, in a horizontal direction, and was reflected at right angles by a mirror into the manometer vessel.

We found that the light had no action at 20° and pH 6·5 with the liquid obtained on centrifuging, not even after the addition of catechol or of catechol and hydroquinone. On the other hand, the light was effective in the sediment obtained on centrifuging only when catechol or catechol and hydroquinone had been added. The light reduced the oxygen uptake. In Fig. 36

an experiment using catechol, and in Fig. 37, one with catechol plus hydroquinone are illustrated. The arrangement in Fig. 37 is preferable as it shows the constancy of the dark reaction. It is of theoretical importance, however, that light action took place when catechol had been added without hydroquinone.

It is to be noted that the sediment suspensions must be freshly prepared from the leaves and the pH must be 6·2 to 6·5.

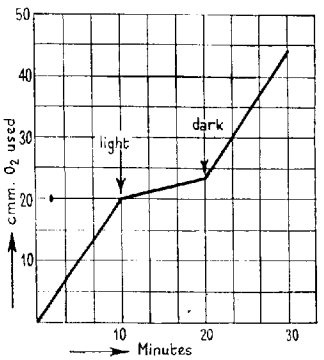


FIG. 37. Action of light on the hydroquinone oxidation in green sediments. 0·2 mg. catechol, 5·0 mg. hydroquinone, 2 cm.^{3} suspension. 20°. pH 6·5. Air in gas space.

At pH 7·4 we observed no light action, and this is remarkable since the oxygen uptake in the hydroquinone oxidation in the dark is the same at pH 6·4 as at pH 7·4. Further, we found no light action when the suspension had been kept 24 hours in ice, whilst the oxygen uptake in the hydroquinone oxidation in the dark was unaffected by the time of keeping. The oxidation of hydroquinone in the dark and the inhibition of the oxidation in the light are therefore processes which differ in their susceptibility.

Since, as was found later, chloride is a 'co-enzyme' for the light action, 0·05 per cent. potassium chloride should be added in all experiments to the green suspensions. In the experiments shown in Figs. 36 and 37 the green sediments were not washed

with water, and so the residual liquid provided enough chloride.

As is shown in Figs. 36 and 37, the oxidation of catechol and hydroquinone is inhibited by light. The simplest explanation of this is that on exposure the reverse reaction takes place to that going on in the dark. That is, in light, quinones are being reduced by the green sediments with evolution of oxygen:

$$2\ o\text{-quinone} + 2H_2O = 2\ \text{catechol} + O_2,$$
$$2\ p\text{-quinone} + 2H_2O = 2\ \text{hydroquinone} + O_2.$$

It will be shown in the following that this explanation is correct.

4. Granule suspensions

The green sediments with which the experiments described above were carried out consisted of intact chloroplasts mixed with granules. A more homogeneous material is obtained by grinding the green sediments with glass balls in closely fitting tubes. In this way all the chloroplasts are broken down to granules, which have about one-tenth of the diameter of the chloroplasts, without the photochemical activity of the material being impaired.

In order to separate the liquid completely we washed the granules three times with water and centrifuged with cooling at a speed corresponding to 22,000 times gravity, the sediment each time being treated with the glass balls and made up with water to the original volume (press juice volume). As starting material for obtaining granule suspensions we used spinach leaves in spring and early summer. From the end of July sugar-beet leaves were used until the weather became frosty. It appeared to make no difference whether the leaves were freshly picked or had been kept for a few days at $+5°$.

The complete process for obtaining the granule suspensions is therefore: spinach or sugar-beet leaves put through a mincer → leaf pulp pressed through cloth → pressed fluid centrifuged at 1,200 times gravity for five minutes → sediment discarded → liquid centrifuged for 10 minutes at 22,000 times gravity with cooling → sediment ground up with glass balls → made up with water to the original press juice volume → washed three times

in the centrifuge with water under cooling → finally made up with water to one-third of the press juice volume → chlorophyll content of the suspension determined → suspension diluted with water so that 1 cm.³ contained 0·9 mg. (1 micromole) chlorophyll.

For the determination of the chlorophyll, a test portion of the suspension was centrifuged at 22,000 times gravity, the sediment triturated with methanol and again centrifuged. In the clear methanol solution we determined the absorption for wavelength 578 mμ, and calculated from that, using the absorption coefficient

$$\beta = 17\left[\frac{\text{cm.}^2}{\text{mg. chlorophyll}}\right],$$

the concentration c of the chlorophyll from the light absorption $\frac{i}{i_0}$ for the layer of thickness d_{cm}.

$$\frac{i}{i_0} = e^{-\beta c d};$$

$$c = \frac{\ln i_0/i}{17d}\left[\frac{\text{mg. chlorophyll}}{\text{cm.}^3}\right].$$

The photochemical activity of the granule suspension decreased on keeping in the ice box. To stabilize it we added one-tenth of its volume M/2 phosphate, pH 6·21 and one-tenth of its volume 0·5 per cent. KCl. In this medium the decrease in activity at 5° amounted to about 15 per cent. in 24 hours.

In order to obtain material for the winter months, we tried to prepare active dry preparations. On freeze drying, the granule suspensions lost about half of their photochemical activity, and on keeping the dry powder the activity further decreased. No better results were obtained by freeze drying the whole leaves. Fresh material must therefore be used.

W. Menke† determined the composition of the chloroplasts from which the granules were obtained. He found 48 per cent. protein, 37 per cent. lipins, and 8 per cent. ash. Included in the lipins was 8·6 per cent. chlorophyll. In the granules we found:

9 per cent. Chlorophyll,

† W. Menke, *Zeitschr. f. physiolog. Chem.* **257**, 43 (1938); **263**, 100 (1940).

3 per cent. Ash,

0·3 per cent. Phosphorus, i.e. 1 mole P per mole chlorophyll,

0·1 per cent. Iron, i.e. 0·18 mole iron per mole chlorophyll,

0·016 per cent. Manganese, i.e. 0·029 mole manganese per mole chlorophyll,

0·0068 per cent. Zinc, i.e. 0·010 mole zinc per mole chlorophyll.

5. Photochemical reduction of quinone

The purest quinone commercially obtainable contains an impurity which is dark coloured and which is poisonous. We removed this by distilling the quinone in steam according to the method of Vanino. The bright-yellow crystals which solidified in the condenser were dried quickly in vacuum and were dissolved without warming to give a 1 per cent. solution. When water is used as solvent the bright-yellow solution quickly darkens and contains a substance which reduces or completely destroys the photochemical activity of the granules. This substance, the formation of which is not caused by the action of air, is not formed if the quinone is dissolved in N/100 sulphuric acid. The latter was therefore used as solvent. Only if these precautions are taken would it be possible to repeat the following experiments.

If quinone is added to a granule suspension which has been saturated with argon at pH 6·5, there is no action in the dark (only traces of carbon dioxide are evolved). Exposure to light from a metal-filament lamp causes evolution of oxygen, the more intense the light the greater the evolution, till the quinone has been used up. 80 per cent. to 90 per cent. of the oxygen required by the equation

$$2 \text{ quinone} + 2H_2O = 2 \text{ hydroquinone} + O_2$$

is then evolved (Fig. 38). The reaction proceeds very slowly or not at all at pH 7·4. The suspension should not be exposed to light before the addition of the quinone. Exposure in the absence of a photochemical substrate is harmful to the granules. The experiment illustrated in Fig. 38 was supplemented by the following tests.

1. The granule suspension was exposed to light as described, but in the absence of quinone. No pressure change took place.

2. With yellow phosphorus in the inner vessel no pressure change was observed on exposure, thus proving that the gas was oxygen.

3. When 10 mg. Na_2SO_4 and 0·1 c.c. N/10 H_2SO_4 were added to 2 c.c. of the granule suspension and the mixture centrifuged,

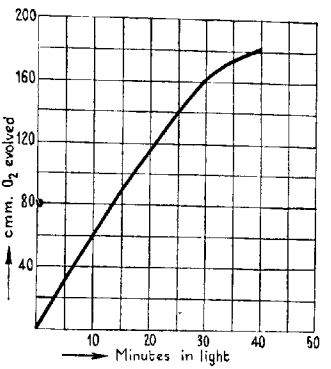


FIG. 38. Photochemical reduction of quinone by green granules. 2 cm.³ granule suspension containing 0·9 mg. = 1 micromole chlorophyll. M/20 phosphate; pH 6·5; 0·05% KCl; 20°; gas space argon. At t_0 2 mg. quinone = 18·5 micromoles quinone in 0·2 cm.³ N/100 H_2SO_4 added and exposed to light.

a clear liquid was obtained. The quinone in this was titrated iodometrically by the method of Valeur.† When the experimental solution containing quinone had been exposed to light till the oxygen evolution had ceased, the quinone was shown to have disappeared, whilst in the control experiment carried out in the dark, it could be recovered completely.

Corresponding experiments with *o*-benzoquinone could not be carried out owing to the instability of this substance, but *β*-naphthoquinone sulphonic acid, a stable *o*-quinone, was reduced by the green granules on exposure, with the production of oxygen. In view of this, and because the oxidation of catechol by the green granules is inhibited by light, it must be assumed

† Valeur, *Compt. Rend.* **129**, 552 (1899).

that *o*-benzoquinone like *p*-benzoquinone is reduced with evolution of oxygen.

It is to be expected that any respiration, the oxygen transporting enzyme of which is a phenol oxidase, will be inhibited by light, for Raper† has shown that *o*-quinones are formed as intermediate products. On the other hand, in spite of repeated experiments, we have never observed that the respiration of green granules was inhibited by light in the absence of catechol.

6. Reaction equation

The following equation for the photochemical reduction of quinone:

$$2 \text{ quinone} + 2H_2O = 2 \text{ hydroquinone} + O_2 - 52{,}000 \text{ cals.}$$

is an expression of the overall reaction only, and no attempt is made to specify the mechanism. Sensitive enzymes are obviously concerned in the process, since the photochemical activity of the granules decreases to half on warming to 40° for ten minutes.

At 50° the activity disappears entirely after ten minutes. Changes in the chlorophyll or agglutination of the granules do not appear to take place under such conditions.

The above equation necessitates the view that the granules act as catalysts. Each constituent of the granules may react, but no component may be used up. Moreover, each change taking place in the granules must be reversible.

Chlorophyll is not used up in the quinone reduction. If chlorophyll had reacted with the quinone, then in experiment II of Fig. 39, for example, an amount of chlorophyll 92 times that of the original amount would have been built up during the reaction, for in this experiment a weight of granules containing only 0·2 micromole chlorophyll was used, whilst 18·5 micromoles quinone were reduced.

In the same experiment also the more general question as to whether any component of the granules is used up in the photochemical reduction of quinone is answered. In experiment II of Fig. 39 the weights of added quinone and granules were the

† H. S. Raper and co-workers, *Bioch. Journ.* **19**, 84, 92 (1925); **21**, 1370 (1927).

same. If any hypothetical substance reacting with the quinone were to be used up, then the total amount of the granule substance would have to react if this hypothetical substance had the same molecular weight as the quinone, and if its molecular weight were greater than that of the quinone, which would probably be the case, then more than the total granule weight would be required. We therefore consider that the experiment

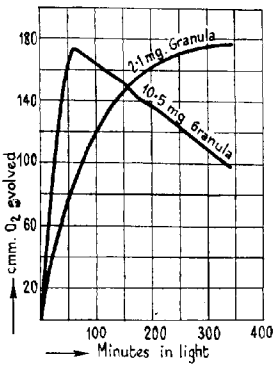


FIG. 39. Photochemical reduction of quinone with varying amounts of granules. 20°; M/20 phosphate; pH 6·5; 0·05% KCl; gas space argon. At t_0 2 mg. = 18·5 micromoles quinone in 0·2 cm. M/100 H_2SO_4 added. Exposed to red light of λ greater than 610 mμ. Curve I: 2 cm.^{3} granule suspension containing 10·5 mg. dry weight and 1 micromole chlorophyll. Curve II: 2 cm.^{3} granule suspension containing 2·1 mg. dry weight and 0·2 micromole chlorophyll.

proves the catalytic nature of the reaction and justifies the above equation.

The fact that the oxygen formed is only 80 per cent. to 90 per cent. of the theory instead of 100 per cent. is due to the oxygen uptake by the granules. Though not negligible this is small with intensive illumination (experiment I, Fig. 39). When the gas space contains air instead of argon, the uptake of oxygen, and therefore the deficit in photochemically produced oxygen, becomes greater.

The oxygen uptake cannot be considered as the reverse reaction to that of the photochemical one. It is not a dark reaction between molecular oxygen and hydroquinone, for the granules

take up oxygen in the absence of hydroquinone, and if hydroquinone is added in the absence of catechol the oxygen uptake is not increased. Moreover, if equal amounts of hydroquinone and quinone are added together the rate and the amount of oxygen evolution are exactly the same as when quinone alone is added (Fig. 40).

The value of 52,000 calories shown in the equation as being required for the production of 1 mole oxygen is arrived at from the amount of light absorbed by the chlorophyll. The intensity

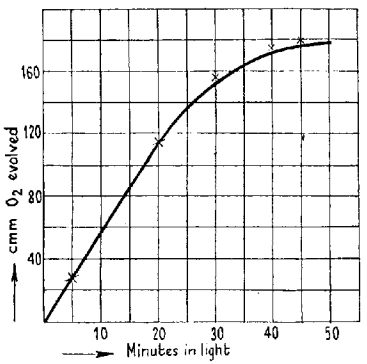


FIG. 40. Photochemical reduction of quinone with addition of hydroquinone. 20°; M/20 phosphate; pH 6·5; 0·05% KCl; gas space argon. 1 micromole chlorophyll per 2 c.c. granule suspension. Continuous line: +2 mg. quinone dissolved in 0·2 cm.³ N/100 H_2SO_4. Crosses: +2 mg. quinone +2 mg. hydroquinone dissolved in 0·2 cm.³ N/100 H_2SO_4.

of the light determines the rate at which the evolution takes place. When the light intensity is kept constant and the concentration of the granules varied, the rate of evolution is greatest when the concentration of the granules is sufficient to produce complete absorption.

Quinone itself absorbs visible light only in the blue region, whilst all regions of the visible spectrum are effective for the quinone reduction. With our technique the light absorption by the quinone in the blue region was negligible in comparison with that by the chlorophyll.

Finally it may be mentioned that up till now only two of the reacting substances have been determined, the quinone used up, and the oxygen evolved. It would be desirable for the sake of completeness to determine also the hydroquinone produced in the reaction.

7. Co-enzyme

When washing the green granules with water in the course of their preparation by centrifuging the activity decreases, and

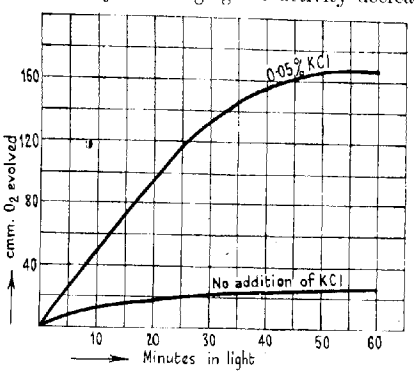


FIG. 41. Reactivation of washed granules by chloride. 1 micromole chlorophyll per 2 cm.³ granule suspension. 20°; M/20 phosphate; pH 6·5; gas space argon. At zero time 2 mg. quinone dissolved in 0·2 cm.³ N/100 H_2SO_4 added.

after three washings it disappears. If, however, cell fluid is again added to the suspension of the granules the activity returns. Addition of cell fluid to the extent of a 500th part of the volume of the granules produces considerable reactivation, and addition of one-tenth of their volume reactivates them completely. Addition of excess of cell liquid is not desirable, since it uses up quinone in a dark reaction. Excess of cell fluid therefore causes a decrease in the photochemically produced oxygen calculated on the basis of the amount of quinone added.

The active substance of the cell fluid is stable to boiling, and is nothing more or less than chloride which is present to the extent of 0·08 mole per litre. The photochemical activity of

washed granules can be regenerated therefore by the addition of potassium chloride (Fig. 41). N/5000 causes appreciable activation, and N/150 makes the reactivation complete.

Although chloride is the natural activator in the cell fluid it can be replaced by other salts such as bromide, iodide, and nitrate. Chloride and bromide have about the same activity, iodide and nitrate are considerably less active. Fluoride, thiocyanate, sulphate, phosphate, and all cations tried were inactive. The light absorbed by the chlorophyll is therefore only effective in the presence of certain anions—a finding which shows how rash all previous theories of the mechanism of carbon dioxide assimilation have been.

When chloride activates it probably does not react with the quinone, as we have proved that the photochemical reduction of ferric salts discovered by Hill† likewise does not take place in the presence of washed granules, but only after addition of chloride. Instead of ferric oxalate we used potassium ferricyanide as the iron salt, and the photochemical reduction of this substance by the green granules, like that of quinone, can be observed and investigated by manometric determination of the oxygen evolved. The pH was 6·1 to 6·5. The phosphate buffer had to be sufficiently strong to prevent the acidity produced in the reaction

$$Fe^{+++} + H = Fe^{++} + H^{+}$$

from lowering the pH below 6·1.

8. Narcotics

Octyl alcohol in saturated solution inhibits completely the photochemical activity of the granules. Addition of 0·1 mg. phenylurethane to 1 cm.^{3} of the granule suspension inhibits to the extent of 50 per cent. The concentration of the phenylurethane in the suspension thus amounts to

$$6{\cdot}1 \times 10^{-4} \text{ mole/litre.}$$

This is the half-value concentration‡ for the inhibition of carbon dioxide assimilation by intact Chlorella.

† Robert Hill, *Proc. Royal Soc.* B **127**, 192 (1939), also introduction to this chapter.

‡ O. Warburg, *Bioch. Zeitschr.* **100**, 230 (1919), also Chapter II of this book.

When, therefore, the cell structure and the chloroplast structure are destroyed, the green granules still retain the substances necessary for the photo-reaction as they are *in vivo*. Enzymes, both soluble and insoluble, are not inhibited by saturated solutions of octyl alcohol and phenylurethane added so as to produce concentrations ten times greater than the above half-value concentration.

9. Phenanthroline

Substances which react specifically and reversibly with heavy metals, such as hydrogen cyanide and cysteine, are oxidized by quinone (with carbon dioxide evolution) at the temperature and pH of our experiments, so that their effect on the quinone reduction cannot be determined. On the other hand, we could use carbon monoxide which does not react under these conditions with quinone. When we filled the gas space in the manometer with carbon monoxide instead of argon we observed the same rate and the same end point for the oxygen evolution. Carbon monoxide does not therefore react with the catalysts of the photo-reaction.

Phenanthroline prepared in 1898 by Fritz Blau† is another heavy metal complex-forming substance stable to quinone. Blau found that this compound forms stable complexes with iron, nickel, cobalt, and zinc, but not with manganese. In each case three molecules of phenanthroline are combined with the heavy metal ion.

Phenanthroline inhibits the photochemical reduction of quinone by green granules at very low concentration (Fig. 42). For example, if 0·02 mg. (0·085 micromole) phenanthroline hydrochloride (molecular weight, 235) are added to 2 cm.^{3} of the granule suspension containing 1 micromole chlorophyll, the inactivation is 50 per cent. Twice this amount of phenanthroline produces complete inactivation.

This amount producing complete inactivation‡ could therefore only combine with a small fraction of the chlorophyll

† Fritz Blau, *Monatshefte für Chemie* (1898).

‡ Calculated for the metal contents of the granules as given in section 4 of this chapter.

whether it is assumed that one or three molecules of the substance react with one of chlorophyll. The cause of the inhibition cannot therefore be a reaction with chlorophyll.

Again, the amount of phenanthroline producing complete inhibition would not be sufficient to combine with all the iron in the granules on the assumption that three molecules of phenanthroline combine with one atom of iron. On the other

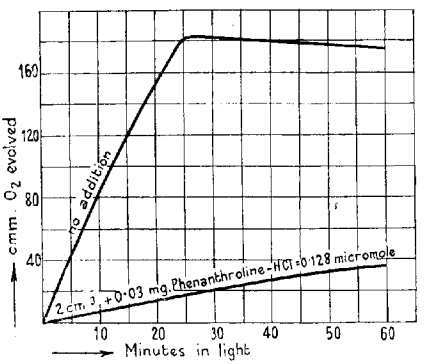


FIG. 42. Inhibition of the photochemical quinone reduction by phenanthroline. 2 cm.³ granule suspension containing 1 micromole chlorophyll; M/20 phosphate; pH 6·5; 0·05% KCl; 20°. Gas space argon. At zero time 2 mg. quinone in 0·2 cm.³ N/100 H_2SO_4 added.

hand, this amount of phenanthroline would suffice to combine with all the zinc in the granules. Manganese need not be considered, because, as has been shown above, this metal does not form stable complexes with phenanthroline.

The phenanthroline inhibition is reversible. If the extent of the inhibition is first determined, and then excess of zinc sulphate is added (from a second side tube), the rate of oxygen evolution rises again to the uninhibited value. Ferrous sulphate also reactivates, but not so completely as the zinc salt (Fig. 43). When the metal salt is first mixed with the phenanthroline and then the granules are added, not only zinc, but also salts of divalent iron, cobalt, and nickel protect the granules completely from the effect of phenanthroline. Thus only the free phenanthroline possesses the inhibitory action.

In the reactivation by metal salts it is to be noted that the amounts of the metal used must be much greater than correspond to the ratio 3 phenanthroline:1 metal. In Fig. 43, for example, the ratio is ten times greater than this. It cannot be concluded therefore that the reactivating metal salts are activators of the photo-reaction. The reactivations show only that the

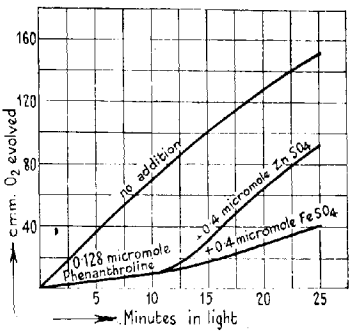


FIG. 43. Inactivation of the granules by phenanthroline, and reactivation by zinc sulphate or ferrous sulphate. Quantities as in Fig. 42.

phenanthroline inhibits the reaction reversibly and that the phenanthroline inhibits only when it is in the free state, and not when bound to metal in the granules. This obviously indicates that the component of the granules with which the phenanthroline reacts is a heavy metal, and it follows from what has been said above that this component can only be zinc or a part of the iron in the granules. The view that the phenanthroline inhibition may possibly be a narcotic inhibition cannot be upheld owing to the very small half-value concentration which is less than $4{\cdot}2 \times 10^{-5}$ mole/litre.

Phenanthroline also inhibits in very low concentration the photochemical reduction of carbon dioxide by surviving Chlorella. Phenanthroline does not inhibit the catalase in green granules and this, as with all catalases, is inhibited by cyanide. The heavy metal of the green granules which we assume reacts with the phenanthroline cannot therefore be the heavy metal of the granule catalase.

10. Sensitivity towards acid

When one-fiftieth of its volume of 2 M acetate buffer of pH 4 is added to an aqueous suspension of the granules containing 1 micromole chlorophyll per cm.^{3}, the pH becomes about 4·2. If the solution is centrifuged and the sediment of granules is suspended in M/20 phosphate of pH 6·5 containing potassium chloride, the photochemical activity on addition of quinone is almost zero. Addition of the supernatant liquid to the inactive granules does not bring back the photochemical activity.

The supernatant liquid contains two-thirds of the zinc in the granules, but only one-fiftieth of the iron. It also contains a substance fluorescing in the ultra-violet and having absorption bands at 270 and 340 mμ. The absorption in these regions of the spectrum was so great that it could be measured in a layer of the liquid 1 cm. thick. It is to be noted that the granules must be washed with water before the addition of the acetate buffer until the washings show no ultra-violet absorption.

11. Surviving Chlorella

Since quinone permeates into living cells, in view of what has been reported in the previous chapters, it was to be expected that surviving green cells would photochemically reduce quinone like the separated green granules. For the sake of completeness the results of an experiment with surviving Chlorella are recorded. The technique was the same as that used with the separated granules (Fig. 44).

In this experiment we found an important difference in the behaviour of the Chlorella as compared with the green granules. Chlorella in the presence of quinone evolved considerable amounts of carbon dioxide in the dark. In five minutes this amounted to about one-fiftieth of the volume of the Chlorella. In comparison with the photochemical oxygen evolution, which, as can be seen from Fig. 44, amounted to 1/2 to 1/1 of the Chlorella volume, this dark reaction is negligible. It, however, becomes relatively greater as the light intensity decreases, and can, therefore, with weak illumination become greater than the oxygen evolution. At low light intensities it could possibly,

therefore, be an intermediate product of the quinone reduction

$$2 \text{ quinone} + H_2O + CH_2O = 2 \text{ hydroquinone} + CO_2$$
$$CO_2 + H_2O = CH_2O + O_2$$
$$2 \text{ quinone} + 2H_2O = 2 \text{ hydroquinone} + O_2.$$

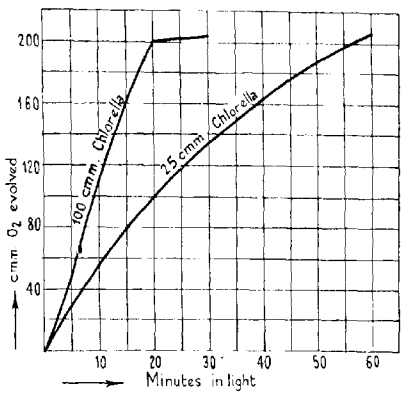


FIG. 44. Photochemical reduction of quinone in surviving Chlorella. 2 cm.³ cell suspension containing 100 mm.³ or 25 mm.³ cells. 20°; argon; M/20 phosphate; pH 6·5; 0·05% KCl. At zero time 2 mg. = 18·5 micromoles quinone added.

Under these circumstances the photochemical quinone reduction might be the result of a dark and a light reaction which have been long recognized as taking place.

In experiments with the cell-free granules, however, the formation of carbon dioxide as an intermediate product is excluded, because the separated granules are incapable of reducing carbon dioxide photochemically. In addition to the many advantages which the separated granules possess over surviving cells as experimental material, it can also be said that the granules give less equivocal results.

CHAPTER XXI

THE QUANTUM REQUIREMENT OF CARBON DIOXIDE ASSIMILATION

This work completed in the spring of 1945 is as yet unpublished

ACCORDING to Robert Emerson† when green algae are exposed to light, carbon dioxide is actually evolved in the first few minutes and not absorbed. This photochemical evolution of carbon dioxide was claimed to be considerable. According to Emerson it had been previously reckoned as oxygen in all manometric determinations of carbon dioxide assimilation and was responsible for the yields being one-fifth too high. Accordingly the photochemical yield in carbon dioxide assimilation was not really 0·25 as had been thought since 1923, but rather 0·083. On this basis the quantum requirement for the reduction of a CO_2 molecule would not be 4 but 12.

1. Refutation of Emerson's views

I disagree with Emerson's findings in two respects. Firstly, according to Emerson the pressure changes, irrespective of their origin, were greater at the beginning of the exposure than later. Secondly, he found that the assimilation quotient CO_2/O_2 was different at the start from what it was later; actually it was markedly positive at the beginning, whilst it eventually became negative, and reached a value of nearly -1. This reversal of effect took place during the first ten minutes of the exposure.

To check this finding I first arranged for F. Kubowitz to repeat the 1923 experiments with a modified technique which allowed of the pressure changes being determined at short time intervals. In these experiments we used Chlorella in Knop solution saturated with 10 per cent. CO_2 and 90 per cent. O_2 by volume, rotating circular manometric vessels and differential manometers arranged so that the pressure changes could be read without stopping the rotation.

For checking the second part of Emerson's work I developed

† Cf. section 9, Chapter XII.

a manometric method by which the assimilation quotient CO_2/O_2 could be determined at time intervals of 5 to 10 minutes.

In order to be quite clear on the problem, it should be remembered that the photochemical yield in the carbon dioxide assimilation is dependent on both internal and external conditions. There are, therefore, many 'yields', but as in thermodynamics, in this case only the maximum yield for the energy change is of scientific interest.

The conditions of culture of the cells is a decisive factor in the investigation of the problem. Dead cells give a photochemical yield of zero. Pure cultures may be pure in respect of their origin, but may not be pure in respect of function. Usually we have a mixture of dead and living, or functioning and non-functioning cells, and if it is required to measure the maximal function of the cells there is no other way than to vary the conditions of culture till the function can be no further increased.

2. The course of the pressure changes with time

In Figs. 45 to 48 the course of the pressure changes with time, using the 1923 method, is graphically illustrated. As can be seen, the pressure changes in the dark give a straight-line graph. On exposure, after a short transition period, a straight line is again obtained. The slope depends on the light intensity. We have varied the light intensities within a factor of 9, and have chosen them so that the pressure changes remained negative at low intensities and weakly or strongly positive at high intensities.

From the graphs it can be further seen that usually the pressure changes in the transition periods are such as would be expected from gas equalization conditions. On exposure to light the dark reaction persists for a few minutes, and on darkening, the light reaction likewise goes on for a short time (non-continuous part of the curves in Figs. 45 to 47). Thus in the first few minutes of darkness we obtain pressure changes corresponding to those missing in the first few minutes of exposure. Under these conditions it is immaterial how the yields

are determined, whether from the stationary light and dark values, or from the total experimental times including both transition periods. The results of both methods of calculation are given; within the limits of error of the determinations they are the same.

Fig. 48 only shows disagreement. The pressure changes

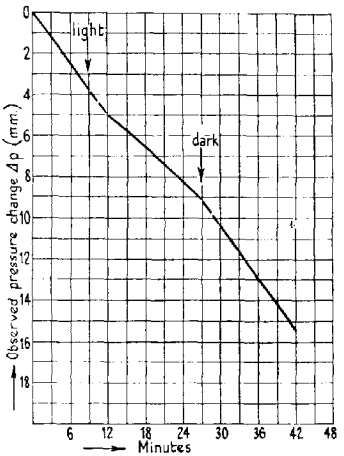


FIG. 45. 10°. 578 mμ. $K_{O_2} = 5{\cdot}40$ mm.^{2} $J = 0{\cdot}158$ micromole quantum/min. $1/\phi$ calculated from the stationary states: 3·96. $1/\phi$ calculated from the total experimental time: 3·60.

on darkening in this case are those to be expected from the gas equalization conditions, but the pressure changes in the first minutes of exposure are the opposite from those to be expected; they are greater than those in the stationary state following. In this case it is not immaterial how the yields are calculated. A higher quantum requirement is obtained from the light and dark values than from the total time of experiment.

This is the 'Emerson effect'. We have observed it only occasionally, and then only when high light intensities were used. It is probably due to the pressure variations resulting

from bubble formation and the conditions favourable to this are when negative pressure changes suddenly change to positive, as happens when high light intensities are being used. In our new technique—the technique used for γ determinations—we

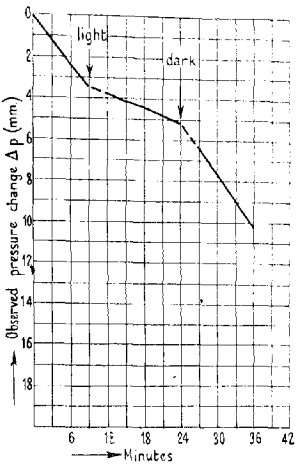


FIG. 46. 10°. 578 mμ. $K_{O_2} = 5{\cdot}40$ mm.² $J = 0{\cdot}33$ micromole quantum/min. $1/\phi$ calculated from the stationary states: 4·5. $1/\phi$ calculated from the total experimental time: 4·6.

have never observed either bubble formation, or, at any time, the Emerson effect.

3. The photochemical yield

If it is desired to calculate the photochemical yield from the pressure changes and the absorbed light energy, the assimilation quotient

$$\gamma = \frac{CO_2}{O_2}$$

must be known, since it appears in the formula†

$$K_{O_2} = \frac{k_{CO_2} \times k_{O_2}}{k_{CO_2} + \gamma k_{O_2}}.$$

† O. Warburg, *Bioch. Zeitschr.* **152**, 51 (1924).

As is shown in the following section,

$$\gamma = -0{\cdot}93.$$

We have used this value to calculate the vessel constant† K_{O_2} which is given in the legends to Figs. 45 to 48.

The light intensities J are also noted. These values correspond

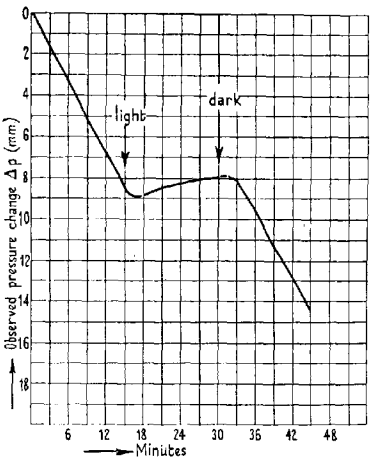


FIG. 47. 10°. 578 mμ. $K_{O_2} = 5{\cdot}40$ mm.² $J = 0{\cdot}75$ micromole quantum/min. $1/\phi$ calculated from the stationary states: 5·03. $1/\phi$ calculated from the total experimental time: 4·70.

to the intensities of the absorbed light, since in all cases there was complete absorption.

If J is expressed as micromole quanta/minute, then in the calculation of ϕ, the photochemically evolved oxygen must also be expressed in micromoles. The formula for the calculation of the yield is, therefore,

$$\phi = \left[\left(\frac{\Delta p}{\Delta t}\right)_{\text{light}} - \left(\frac{\Delta p}{\Delta t}\right)_{\text{dark}}\right] \frac{K_{O_2}}{22{\cdot}4} \frac{1}{J}.$$

All the quantities in this formula are given in the curves and

† O. Warburg, *Bioch. Zeitschr.* **152**, 51 (1924).

legends of Figs. 45 to 48, so the various yields corresponding to the different parts of the curves can be calculated.

Again, as in 1923, we obtain a quantum number 4 which, with increasing light intensity, tends to increase for the reasons given above.

However, the quantum number 4 is not, as has often been

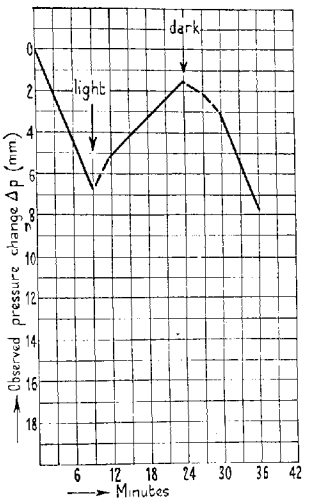


FIG. 48. 10°. 578 mμ. K_{O_2} = 5·40 mm.² J = 1·42 micromole quantum/min. $1/\phi$ calculated from the stationary states: 5·56. $1/\phi$ calculated from the total experimental time: 4·45.

erroneously stated, the thermodynamically possible limit. It is the experimental limit which we previously obtained and which could not be lowered by any variation in the conditions of culture. I am satisfied that the quantum number 4 is correct for the physical or chemical mechanism of the photo-reaction.

Note 1. Figs. 45 to 48 also provide some evidence in relation to the frequently discussed question as to whether in calculating the yield it is correct to subtract the 'dark' pressures from the 'light' pressures, i.e. whether our assumption that the respiration on exposure to light (which cannot be determined) is the

same as that in the dark. An examination of the figures shows how the ratio respiration: photo-action differed in the four experiments. One could hardly have found almost the same yields from the results of the four experiments if the principle of the method had been at fault.

Note 2. In contrast to the assimilation quotient, the respiratory quotient which we determined gasometrically† in 1922

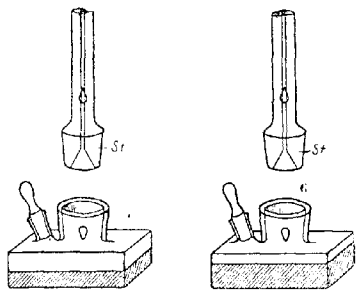


FIG. 49. Manometer vessel for the determination of γ. The figure shows the different fillings of the vessels by which the ratio gas space: liquid volume was altered.

and found to be -1 does not come into the calculation of the photochemical yield. If the pressure changes found in the dark have been subtracted from those taking place on exposure, all the dark reactions are eliminated, and it is immaterial what happens chemically in the dark reactions.

4. Principle of the γ determination

Chlorella suspended in Knop solution and saturated with a mixture of 10 per cent. carbon dioxide and 90 per cent. oxygen by volume was introduced into a manometer vessel of the type shown in Fig. 49. The total volume of the vessel was 14·47 cm.^{3}, and the area of the base about 8 cm.^{2} The vessel was connected with an ordinary Haldane–Barcroft blood-gas manometer and shaken with a to-and-fro movement as in our most recent

† O. Warburg and E. Negelein, *Zeitschr. f. physikalische Chemie*, **102**, 236 (1922).

method.† Red light 635–675 mμ was used. This came horizontally through a window in the thermostat and was reflected at right angles by a mirror into the vessel. The cell suspension was concentrated enough to absorb the light completely.

The pressure changes occurring when the vessel was shaken were read off at intervals of five minutes, first after the addition of 5 cm.³ of cell suspension, and then after a further addition of 3 cm.³ of Knop solution, so that the volume of liquid containing the same amount of cells was increased from

$$v_F = 5 \text{ to } v_F = 8 \text{ cm.}^3$$

If the pressure changes on exposure for the same time intervals were

$$H \text{ for } v_F = 5 \text{ and } H' \text{ for } v_F = 8,$$

then applying our 1924 equation‡ the photochemically produced oxygen would be

$$x_{O_2} = H \frac{k_{CO_2} \times k_{O_2}}{k_{CO_2} + \gamma k_{O_2}} \tag{1}$$

$$x'_{O_2} = H' \frac{k'_{CO_2} \times k'_{O_2}}{k'_{CO_2} + \gamma k'_{O_2}} \tag{2}$$

and since for the same amount of cells and at the same light intensity

$$x_{O_2} = x'_{O_2} \tag{3}$$

it follows from (1) and (2) that

$$H \frac{k_{CO_2} \times k_{O_2}}{k_{CO_2} + \gamma k_{O_2}} = H' \frac{k'_{CO_2} \times k'_{O_2}}{k'_{CO_2} + \gamma k'_{O_2}}. \tag{4}$$

In this equation the only unknown is γ. The equation is solved for γ, or more conveniently, the values of the ratio H'/H are calculated for different values of γ and the values are recorded in a table from which the value of γ corresponding to the experimentally determined ratio H'/H is selected. For our experimental conditions

$v = 14{\cdot}47$ cm.³, $v_F = 5{\cdot}0$ cm.³, $v_F = 8$ cm.³,

and the temperature 20°,

† Cf., e.g., O. Warburg, *Bioch. Zeitschr.* **100**, 230 (1919).
‡ O. Warburg, ibid. **152**, 51 (1924).

we then obtain

γ	H'/H
−1·3	4·75
−1·2	3·36
−1·1	2·72
−1·0	2·35
−0·9	2·12
−0·8	2·04
−0·7	1·82

5. Determination of γ

The assumption is made that the conditions are such that

$$x_{0_2} = x'_{0_2}. \tag{3}$$

This means that complete light absorption must take place not only when the vessel is stationary, but also when the thickness of the cell suspension layer is varying during the shaking process.

Two essential conditions arise in this connexion. First, the light absorption must be more than just complete. We chose a region of the spectrum about 655 mμ for which the light absorption coefficient of the chlorophyll is about $72 \frac{\text{cm.}^2}{\text{mg. chlorophyll}}$. The surface density of the chlorophyll in the vessel was about 0·125 mg. chlorophyll/cm.^{2}, so that in the stationary vessel the light absorption amounted to

$$\frac{i}{i_0} = e^{-\beta cd} = e^{-72 \times 0{\cdot}125} = e^{-9} = 0{\cdot}00012.$$

Secondly, it was necessary for the movement of the vessel to be regular, with a small stroke, so that the thickness of the layer did not change too much, and the movement had to be fast enough so that in spite of the small stroke the agitation of the liquid was sufficient to enable the gas exchange to take place.

Actually we made certain that the above condition (3) had been realized by varying the shaking of the vessel and the volume of cell suspension introduced.

In these experiments there was fortunately never any frothing. This was the difficulty associated with our earlier method for the determination of ϕ in which the cell suspension was given a rotatory movement. It is recommended therefore that square

vessels instead of round ones, and a to-and-fro, instead of a rotatory movement, be used in all determinations of ϕ.

6. Results

In the first and second ten-minute periods of exposure we found

$$\gamma = -0{\cdot}93.$$

This value agrees within the experimental error with our previous value $\gamma = -0{\cdot}91$ which we obtained with the gasometric method† using longer time intervals and higher light intensities. The simplest and most probable view, that the assimilation quotient is independent of the exposure time and the light intensity was again shown to apply in this case.

If Emerson had been correct we should have found, as can be calculated from his quantum yield, a value for $\gamma = +4{\cdot}0$ instead of $-0{\cdot}93$, whilst deviations from our value only within the limits $\gamma = -0{\cdot}8$ to $\gamma = -1{\cdot}2$ could be admissible.

Example. In the vessel: 100 mm.^{3} Chlorella pyrenoidosa suspended in Knop solution saturated with 10 per cent. CO_2 and 90 per cent. O_2 by volume. Red light, 635–675 mμ. Temperature 20°. For the first 10 minutes of exposure we found

$$H = 11 \text{ mm.}, \; H' = 24 \text{ mm.}, \text{ therefore } H'/H = 2{\cdot}18;$$

and in the second 10 minutes

$$H = 12 \text{ mm.}, \; H' = 26 \text{ mm.}, \text{ therefore } H'/H = 2{\cdot}17.$$

From these values, using the table (section 4) for both time intervals,

$$\gamma = -0{\cdot}93.$$

7. A very simple method for the determination of the photochemical yield

If the energy of the light used in the manometric determinations of γ is measured, the photochemical yield ϕ can also be obtained for each determination.

Instead of using the bolometric method, which involves a considerable amount of apparatus, I should like to suggest a chemical method. Chlorophyll, dissolved in dioxan, on being

† O. Warburg and E. Negelein, *Zeitschr. f. physikalische Chemie*, **102**, 236 (1922).

exposed to light transports oxygen to suitable acceptors with a photochemical yield $\phi = 1$. If, therefore, into a manometer vessel we put some chlorophyll in a layer sufficiently dense to absorb completely the irradiating light, we can obtain the light energy from the oxygen uptake read off from the manometer using the simple relationship

quanta irradiated = molecules oxygen transported.

The complete directions for the determination of the photochemical yield in the carbon dioxide assimilation are therefore:

A small vessel of the type shown in Fig. 49, having a capacity of about 15 cm.^{3} and base area of 8 cm.^{2}, is fitted with an ordinary Haldane–Barcroft blood-gas manometer. A parallel beam of red light 635 – 675 mμ obtained from a metal-filament lamp using copper sulphate and a red filter is directed to the bottom of the vessel at right angles to the base. The light is completely absorbed by the chlorophyll in the cells or in solution. Since the light beam has a smaller cross-section than the base of the vessel, no light is lost by scattering through the sides of the vessel.

Into the vessel, which is shaken quickly, but with a small stroke, in a thermostat, the following are introduced one after the other:

1. 1 mg. ethyl chlorophyllide and 58 mg. allyl thiourea dissolved in 5 cm.^{3} of pure dioxan. The gas space contains pure oxygen.
2. 100 mm.^{3} Chlorella suspended in 5 cm.^{3} Knop solution. Gas space, 10 per cent. CO_2 and 90 per cent. O_2.
3. 100 mm.^{3} Chlorella suspended in 8 cm.^{3} Knop solution. Gas space, 10 per cent. CO_2 and 90 per cent. O_2.

If the oxygen transported by the chlorophyll in dioxan, and the oxygen evolved by the algae are determined in turn for the same light intensity and exposure period, the photochemical yield in the carbon dioxide assimilation

$$\phi = \frac{\text{oxygen evolved by the algae}}{\text{oxygen transported by the chlorophyll}}.$$

This determination of ϕ is so simple that it could be introduced into an elementary course in physiology.

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